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(57) Abstract

Nitric oxide, neuronal nitric oxide synthase, neuronal nitric oxide synthase binding proteins, their inhibitors and a method of use of neuronal nitric oxide synthase, its binding proteins and their inhibitors for diagnosis and treatment of muscular dystrophy, stroke and other neurodegenerative diseases. A diagnostic assay for detection of absence of dystrophin or its mutated forms, neuronal nitric oxide synthase or its binding proteins. A method for treatment of muscular dystrophies by restoration of a functional dystrophin molecule in dystrophic muscles using gene therapy. Neuronal nitric oxide binding proteins PSD-95 and PSD-93 involved in management of stroke and other neurodegenerative diseases. The cloning and expression of the neuronal nitric oxide synthase binding proteins.

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MUSCULAR DYSTROPHY, STROKE, AND NEURODEGENERATIVE DISEASE DIAGNOSIS AND TREATMENT

BACKGROUND OF THE INVENTION

Field of Invention

This invention concerns nitric oxide, neuronal nitric oxide synthase, neuronal nitric oxide synthase binding proteins, their inhibitors and a method of use of neuronal nitric oxide synthase, its binding proteins and their inhibitors for diagnosis and treatment of muscular dystrophy, stroke and other neurodegenerative diseases.

In particular, one aspect of the invention concerns involvement of neuronal nitric oxide synthase and its binding protein α1-syntrophin in muscular dystrophic diseases, and their use in diagnosis and therapy of muscular dystrophies. The invention also concerns diagnostic assay for detection of absence of dystrophin or its mutated forms, neuronal nitric oxide synthase or its binding proteins as well as a method for treatment of muscular dystrophies by restoration of a functional dystrophin molecule in dystrophic muscles using gene therapy.

The second aspect of the invention concerns involvement of neuronal nitric oxide, neuronal nitric oxide synthase and its binding proteins PSD-95 and PSD-93 in stroke and other neurodegenerative diseases. The invention also concerns diagnosis as well as prevention and treatment of stroke and the other neurodegenerative diseases. Additionally, invention concerns the cloning and expression of the neuronal nitric oxide synthase binding proteins. Finally, the invention concerns a binding assay for monitoring of binding of nitric oxide binding proteins with neuronal nitric oxide synthase and appropriate synaptic receptors useful for development of compounds for treatment of neurodegenerative diseases.

BACKGROUND ART AND RELATED ART DISCLOSURES

Muscular dystrophy is a debilitating disease caused by a motor dysfunction due to a genetic abnormality resulting in the absence or mutation of the protein dystrophin. Muscular dystrophies consist of a group of inherited diseases characterized by progressive weakness and degeneration of muscle fibers, without evidence of neural degeneration. The group includes dystrophies such as pseudohypertrophic Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy (Leyden-Mobius pelvifemoral type), and facioscapulohumoral (Landouzy-Dejerine) muscular dystrophy.

Duchenne dystrophy is an X-linked recessive disorder caused by a mutation at the Xp21 locus, which results in the absence of the gene product dystrophin, normally localized in the sarcolemma of muscle cells. Becker muscular dystrophy, also a X-linked disorder, is a milder clinical variant of Duchenne dystrophy with the same genomic mutation at Xp21 where patients do not lack dystrophin completely but their dystrophin has an abnormal molecular weight and is somehow dysfunctional.

Duchenne muscular dystrophy is a severe, and ultimately fatal, disease. Duchenne dystrophy patients, typically boys 3-7 years old, experience muscle weakness, waddling gait, toe-walking, lordosis, frequent falls, and difficulty in standing up and in climbing stairs. Progression of the disease is steady and most patients are confined to a wheelchair at age 10 to 12. Few patients survive age of 20 years. Clinical symptoms of Becker muscular dystrophy are less severe; very few patient are confined to a wheelchair and more than 90% of these patients survive.

There is no known diagnostic procedure available for diagnosis, particularly early diagnosis of the disease and its severity, other than muscle biopsy or electromyography. Currently, the diagnosis is based chiefly on clinical signs and on the patient's genetic pedigree. Similarly, there is

no specific therapy for muscular dystrophy other than supervised exercise programs and weight management (The Merck Manual, 16th Ed., 1526 (1992)).

It would be, therefore, advantageous to provide and have available a method for early diagnosis of the muscular dystrophy by using, for example, immunoblotting for detection of dystrophin, syntrophin or nNOS presence or absence in the patient's skeletal muscles, as well as a method for treatment of muscular dystrophy by providing a functional dystrophin molecule or functional fragment thereof or generating means for synthesis of normal nonmutated dystrophin in dystrophic muscles using the method of the invention, including gene therapy.

Dystrophin which has been shown to be missing or mutated in muscular dystrophy physically links the extracellular matrix to the muscle skeleton. Dystrophin is a large intracellular protein containing several defined sequence motifs (Cell, 80:675-679 (1995)). An amino terminal α-actinin-like domain binds to F-actin and is followed by a large rod domain that shares sequence homology with the structural repeats in spectrin. The carboxyl terminus is unique to dystrophin and dystrophin related proteins as this region directly binds to a glycoprotein complex in skeletal muscle. The structural dystrophin glycoprotein complex includes intracellular proteins syntrophins as well as integral membranes proteins, the dystroglycans and sarcoglycans. The absence of dystrophin in Duchenne dystrophy causes a disruption of this complex (Nature, 345: 315-319 (1990)).

Dystrophin was originally identified by positional cloning as the gene product mutated in Duchenne muscular dystrophy (Nature, 323: 646-650 (1986)). Subsequent studies have identified a family of intracellular and transmembrane glycoproteins in a dystrophin-associated complex that links the extracellular matrix with the actin-based cytoskeleton. Recent studies indicate a major role for this complex in neuromuscular development and disease.

α-Dystroglycan, an extracellular glycoprotein linked to dystrophin, serves as a physiologic receptor for agrin, which mediates clustering of acetylcholine receptors (Cell, 77: 663-674 (1994), ibid, 77: 675-686 (1994)). On the other hand, disruption of dystrophin or other proteins in this complex results in muscular dystrophy in both humans and animals (Cell, 80: 675-679 (1995)). Despite these data, it is not known how the dystrophin complex mediates signal transduction, nor is it clear why disruption leads to muscle disease. Indeed, none of the previously identified dystrophin-associated proteins have known catalytic activities.

In addition to this structural role, the dystrophin complex is involved in signalling function in muscle, including regulation of a stretch-activated calcium channel. Signal transduction by the dystrophin complex must be somehow mediated. It has now been discovered that nitric oxide may be that mediator.

Nitric oxide (NO) is a major endogenous mediator involved in diverse developmental and physiological processes (Annu. Rev. Biochem., 63: 175-195 (1994)). In addition to controlling diverse cellular processes, NO also participates in certain pathophysiological conditions. In skeletal muscle NO has been shown to depress the muscle contractile function (Nature, 372: 546-548 (1994)). In the brain, nitric oxide plays important physiological role in neurotransmission and synaptic modulation. In primary cortical cultures, NO mediates glutamate neurotoxicity (PNAS, 88: 6368-6371 (1991)). Neuronal NO production contributes to the development of ischemic brain necrosis (Science, 265:1883-1885 (1994)). A fundamental understanding of NO actions in the brain requires identification of the functional connections of NO synthetic enzyme NNOS with N-methyl-D-aspartate (NMDA) receptors. Deregulation of nNOS in the brain is associated with glutamate type receptor overactivity, of which the NMDA receptor is a member, and contributes to neuronal

damage in animal stroke models (Ann. Neurol., 32: 297-311 (1992)) and, conceivably, nNOS is therefore also involved in stroke and other neurodegenerative diseases in humans.

Thus, it would be important to have a means to prevent development of and/or to treat stroke and the other neurodegenerative diseases by providing inhibitors of neuronal nitric oxide, neuronal nitric oxide synthase or inhibitors of its binding to the NMDA receptors.

Because NO is a short-lived free radical, regulation of signaling occurs largely at the level of NO biosynthesis. Three mammalian nitric oxide synthase (NOS) genes have been identified, and each forms NO from the guanidine nitrogen of L-arginine in a unique cytochrome P-450-type reaction that consumes reduced nicotinamide adenine dinucleotide phosphate.

Specifically, these three NOSs are endothelial (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The nNOS and eNOS enzymes are discretely expressed in specific tissues and rapidly transduce signaling events in a calcium-dependent manner. eNOS activity accounts for endothelium-dependent blood vessel relaxation, while nNOS occurs discretely in a variety of cell types, including neurons, epithelial cells, mesangial cells, and skeletal muscle cells. Inducible iNOS is a calcium-independent form of NOS expressed at highest levels in immunologically activated cells.

Physiological actions for nNOS have been characterized in the peripheral nervous system, where NO functions as a noradrenergic-cholinergic transmitter in numerous pathways, including the gastrointestinal and urogenital tracts.

In addition to transcriptional control, NOS proteins are all regulated by calmodulin (PNAS., USA, 87: 682-685 (1990)), which links NO formation to increases in cellular calcium. Activation of nNOS in neurons is regulated by the steep gradients of calcium that exist in the vicinity of open calcium channels. In many central neurons, calcium influx through the N-methyl-glutamic acid receptor is

selectively coupled to nNOS activity.

A more complex level of regulation is reflected by targeting of NOS proteins to intracellular membranes. This subcellular targeting restricts NO signaling to specific targets. Membrane association of eNOS is mediated by two fatty acid modifications (J. Biol. Chem., 270: 995-998 (1995)). Neuronal NOS lacks consensus sequences for fatty acid modification (J. Neurochem., 62: 1524-1529 (1994)). The majority of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum and specialized electron-dense synaptic membrane structures. In skeletal muscle, nNOS is associated with the sarcolemma (Nature, 372: 546-548 (1994)).

Recent studies have identified nNOS expression at higher levels in human skeletal muscle than in human brain and thus would point toward a role of NO in skeletal muscle (FEBS Lett.,316:175-180 (1993)). In mature skeletal muscle, nNOS is enriched in fast-twitch muscle fibers, where NO opposes contractile force (Nature, 372:546-548 (1994)). It has now been found that the physiological actions of NO in muscle are facilitated by restriction of the nNOS protein to the sarcolemmal membrane.

The sarcolemma of skeletal muscle is a complex structure reinforced by an actin-containing cytoskeleton. In addition to ubiquitous structural elements such as spectrin, skeletal muscle sarcolemma contains a unique network formed around dystrophin and related proteins (Curr. Opin. Cell Biol., 5: 82-84 (1993)).

The N-terminal domain of nNOS is unique to the neuronal isoform and contains a PDZ motif of approximately 100 amino acids that is found in a diverse group of cytoskeletal proteins and enzymes (Neuron, 9: 929-942 (1992)). This domain has now been found to mediate association of nNOS with the dystrophin complex. Therefore it would seem that nNOS must play a distinct role in the muscular dystrophy development and control, and could be advantageously used for early detection of the

dystrophic disease.

It is therefore a primary objective of this invention to provide means for early diagnosis of muscular dystrophies as well as means for developing and identifying means and compounds suitable for treatment of muscular dystrophies, stroke and other neurodegenerative diseases. To this end the invention discloses a role of NO, nNOS and its binding proteins in development or controlling of muscular dystrophy in one aspect, and in prophylaxis, treatment and diagnosis of stroke and other neurodegenerative diseases in another aspect.

All cited patents, patent applications or publications are hereby incorporated by reference in their entirety.

SUMMARY

One aspect of the current invention concerns a function of nitric oxide, neuronal nitric oxide synthase, and neuronal nitric oxide synthase binding proteins in muscular dystrophies.

Another aspect of the current invention concerns a function of nitric oxide, neuronal nitric oxide synthase, and neuronal nitric oxide synthase binding proteins in stroke and other neurodegenerative diseases.

Another aspect of the current invention concerns nitric oxide synthase binding proteins, their cloning and expression.

Another aspect of the current invention concerns identification of two brain proteins, namely, postsynaptic density PSD-95 and postsynaptic density PSD-93 proteins that bind to neuronal nitric oxide synthase.

Still yet another aspect of the current invention concerns a discovery that neuronal nitric oxide synthase is functionally connected to calcium influx through a N-methyl-D-aspartate receptor where, at a receptor synaptic junction, neuronal nitric oxide synthase is enriched with post-synaptic density proteins.

Still another aspect of the current invention concerns identification of inhibitors of nitric oxide synthase

binding proteins.

Another aspect of the current invention concerns identification of a small 9-mer peptide that potently blocks binding of neuronal nitric oxide synthase with post-synaptic density proteins.

Still another aspect of the current invention is a method of use of neuronal nitric oxide synthase, its binding proteins, and their inhibitors, for diagnosis and treatment of muscular dystrophy.

Yet another aspect of the current invention concerns diagnostic assay for detection of absence of dystrophin or its mutated form, as well as a method for treatment of muscular dystrophy by restoration of a functional dystrophin molecule, or a functional fragment thereof in dystrophic muscles using gene therapy.

Still yet another aspect of the current invention concerns a binding assay for monitoring of binding of nitric oxide binding proteins with neuronal nitric oxide synthase and with appropriate synaptic receptors, useful for development of compounds for treatment of stroke and other neurodegenerative diseases.

Still another aspect of the current invention is a method of use of neuronal nitric oxide synthase, its binding proteins and their inhibitors for diagnosis and treatment of stroke or other neurodegenerative diseases.

Still another aspect of the current invention concerns a method for prevention of brain damage due to nitric oxide, by blocking the binding between neuronal nitric oxide synthase and postsynaptic density proteins resulting in uncoupling neuronal nitric oxide synthase from neurotransmitter receptors.

BRIEF DESCRIPTION OF FIGURES

Figure 1 illustrates differential extractability of nNOS and eNOS in skeletal muscle homogenates.

Figure 2 is a schematic alignment of eNOS and nNOS domains showing the extended N-terminus of nNOS containing a PDZ domain.

Figure 3 shows association of nNOS and dystrophin in skeletal muscle in wild-type, *mdx* and NOS knockout skeletal muscle.

Figure 4 shows extraction of skeletal muscle membrane in build-type and *mdx* mice and nNOS displacement from particulate fractions of the *mdx* skeletal muscles.

Figure 5 shows immunofluorescent staining for nNOS of cryostat muscle section from quadriceps of wild-type, *mdx*, homozygous dystrophic and nNOS knockout mice showing that nNOS is selectively absent from sarcolemma of the *mdx* skeletal muscle.

Figure 6 are skeletal muscle cryosections of normal and DMD patients showing nNOS to be absent from sarcolemma of DMD muscle fibers.

Figure 7 is a SDS-PAGE of human skeletal muscle tissue homogenates from three cases of Duchenne muscular dystrophy and from three normal muscle biopsies.

Figure 8 shows localization of nNOS, dystrophin and other dystrophin associated proteins during postnatal development.

Figure 9 are immunofluorescent stained cryosections from mouse quadriceps labeled for nNOS, α1-syntrophin and α-BGT showing localization of nNOS and α1-syntrophin in wild type, *mdx* and transgenic *mdx* mice.

Figure 10 are Western blots of mouse skeletal muscle homogenates showing subcellular distribution of nNOS in transgenic *mdx* mice.

Figure 11 are Western blots of solubilized membranes from mouse quadriceps showing selective interaction of nNOS and α1-syntrophin.

Figure 12 are immunostained cryosections of skeletal muscle sarcolemma in Becker muscular dystrophy patients showing absence of nNOS from skeletal muscle sarcolemma in patients with Becker muscular dystrophy.

Figure 13 is a molecular model of nNOS and NMDA receptor binding PSD-95.

Figure 14 shows alignment of PSD-93 and PSD-95 three PDZ repeats, a SH3 domain and a region homologous to guanylate kinase.

Figure 15 shows expression of PDS-93, PDS-95 and nNOS in a rat brain and E15 embryos.

Figure 16 illustrates PSD-95 colocalization with nNOS in developing neurons.

Figure 17 illustrates nNOS binding to PSD-95 through PDZ motif interaction.

Figure 18 shows alternative splicing of exons 1 and 2 of nNOS.

Figure 19 shows that catalytically active nNOS isoform lacking exon 2 are expressed in the brain in nNOS Δ/Δ.

Figure 20 shows that nNOS isoforms lacking the PDZ motif do not bind to PSD-95 or to brain membranes.

Figure 21 illustrates binding of α1 syntrophin to the N-terminal PDZ containing domain of nNOS.

Figure 22 shows direct binding of nNOS to α1-syntrophin PDZ domain.

DEFINITIONS

As used herein:

"NOS" means nitric oxide synthase, an enzyme that regulates production of nitric oxide.

"NO" means nitric oxide.

"nNOS" means neuronal nitric oxide synthase.

"NMDA" means N-methyl-D-aspartate receptor, which is a glutamate type receptor.

"PSD-95" means postsynaptic density-95 protein, which is present at the brain synaptic junction.

"PSD-93" means post-synaptic density-93 protein, which is present at the brain synaptic junction.

"PDZ" means a N-terminal domain of nNOS, containing a 66-amino acid motif, bearing homology to a heterogeneous family of signaling enzymes localized at cell-cell junctions.

"CAM" means calmodulin.

"FMN" means flavin mononucleotide.

"FAD" means flavin adenine dinucleotide.

"T-SYN" or "SYN-1" means syntrophins.

"DMD" or "DD" means Duchenne muscular dystrophy.

"BMD" means Becker muscular dystrophy.

"*mdx*" or "*mdx* mice" means mice that specifically lack dystrophin due to a nonsense mutation, but express nNOS at near normal levels.

"dy" mouse means a mouse which has severe muscular dystrophy due to an absence of an extracellular matrix protein, merosin, but has a normal distribution of dystrophin at the sarcolemma.

"Syntrophins" means a family of dystrophin-binding proteins which colocalize with nNOS beneath the sarcolemmal membrane.

DETAILED DESCRIPTION OF THE INVENTION

The current invention involves a discovery that nitric oxide, neuronal nitric oxide synthase and neuronal nitric oxide synthase binding proteins are involved in the development and management of a group of muscular dystrophic and neurodegenerative diseases such as stroke. Muscular dystrophic diseases are characterized by the complete absence, or by diminished level of a fully functional dystrophin. Stroke and other neurodegenerative diseases are characterized by overactive N-methyl-D-aspartate receptors linked to nitric oxide formation in neurons.

The invention comprises two parts. The first part is directed to the diagnosis and treatment of muscular dystrophies. The second part is directed to the diagnosis, prophylaxis and treatment of stroke and other neurodegenerative diseases.

I. Diagnosis and Treatment of Muscular Dystrophies

Patients suffering from muscular dystrophy have been known to either lack the protein dystrophin completely or to possess dystrophin which is somehow dysfunctional. It has now been discovered that not only dystrophin but also nitric oxide synthase is absent or its level is lower in

the skeletal muscle of the muscular dystrophy patients.

Neuronal nitric oxide synthase is localized in sarcolemma of fast-twitch fibers and it has now been shown that nNOS partitions with skeletal muscle membranes on account of its association with dystrophin. The dystrophin is associated with intracellular and transmembrane glycoproteins forming a dystrophin-associated complex. The dystrophin complex interacts with a N-terminal domain of nNOS that contains a PDZ motif. Muscles of muscular dystrophy patients show selective loss of nNOS protein and loss of catalytic activity from muscle membrane.

While patients with Duchenne dystrophy lack dystrophin completely and dystrophin of patients with Becker muscular dystrophy is mutated, it has now been found that in both cases there is a loss of nNOS in the skeletal muscle. In healthy individuals, nNOS is concentrated at synaptic junctions at motor endplates of the skeletal muscle where the N-terminus domain of nNOS, which contains a PDZ protein motif, binds to the PDZ motif present in α1-syntrophin. The PDZ domain thus mediates binding of nNOS to skeletal muscle syntrophin, a dystrophin associated protein.

The invention also describes a method of use of nitric oxide, neuronal nitric oxide synthase, its binding proteins and their inhibitors for diagnosis and treatment of muscular dystrophy. A diagnostic assay for detection of absence of dystrophin or its mutated form and a method for treatment of muscular dystrophy by restoration of a functional dystrophin molecule in dystrophic muscles using gene therapy are also disclosed.

Absence or Deficiency in Formation of Dystrophin-nNOS Complex in Muscular Dystrophy

Muscular dystrophies have been characterized by the complete absence of dystrophin in Duchenne dystrophy or by truncated dystrophin in Becker muscular dystrophy. It has now been discovered that additionally, these diseases also lack the normal level of neuronal nitric oxide synthase.

Duchenne Dystrophy and a Function of Neuronal Nitric Oxide Synthase in Skeletal Muscle

Nitric oxide (NO) is synthesized in skeletal muscle by neuronal-type nitric oxide synthase (nNOS), which is localized to sarcolemma of fast-twitch fibers. Synthesis of NO in active muscle opposes contractile force. It has now been shown and described in studies below that nNOS partitions with skeletal muscle membranes owing to association of nNOS with dystrophin, the protein missing in Duchenne muscular dystrophy (DMD). In healthy muscle, the dystrophin complex interacts with the N-terminal domain of nNOS that contains a PDZ motif. In the muscular dystrophy (mdx) mice model and in human DMD skeletal muscle samples, a selective loss of nNOS protein as well as loss of catalytic activity from muscle membranes was found, demonstrating a novel role for dystrophin in localizing a signaling enzyme to the myocyte sarcolemma. Aberrant regulation of nNOS production is therefore suspected to contribute to preferential degeneration of fast-twitch muscle fibers in DMD.

Presence and Absence of nNOS in Skeletal Muscle Sarcolemma

Neuronal NOS is present in cytoskeletal extracts from a healthy skeletal muscle and have been found to be associated with membrane.

In order to elucidate a function of nNOS in muscular dystrophies and to understand the mechanism of membrane association of nNOS, the extractability of nNOS from mouse quadriceps was investigated and compared to extractability of eNOS. Results are seen in Figure 1.

Figure 1 illustrates differential extractability of nNOS and eNOS in mice skeletal muscle homogenates. For these studies, tissue was prepared, extracted and submitted to Western blot analysis as described in Example 1. After subcellular fractionation and 7.5% SDS-PAGE (100 μg protein per lane), nNOS and eNOS were sequentially detected by protein immunoblot. Positions of molecular size markers

are indicated in kilodaltons.

Obtained results, shown in Figures 1A and 1B, show that nNOS is anchored both to microsomal membranes (S2 and S3) and to cytoskeleton (P) whereas eNOS is only found in microsomal membranes (S_2 and S_3).

Figure 1A is a Western blot showing an association on nNOS of an insoluble pellet (P), as well as mouse quadriceps homogenates sequentially extracted as indicated. Western blotting indicates that significant nNOS remains in an insoluble pellet (P) following sequential extraction of mouse quadriceps homogenates with 100 mM NaCl (S_1), 500 mM NaCl (S_2), and 0.5% Triton X-100 (S_3), lower levels of nNOS are present in each of these fractions.

Figure 1B is a Western blot showing association of eNOS with particulate fractions. As seen in Figure 1B, eNOS is found only in membrane-associated fractions but is not present in cytosol and in the insoluble pellet. Probing the same blot with an eNOS monoclonal antibody indicates the eNOS is completely extracted by 500 mM NaCl (S_2) and 0.5% Triton X-100 (S_3).

Results of this study show that nNOS is anchored both to microsomal membranes and to cytoskeleton. Thus, the majority of nNOS protein remained membrane-associated following extensive washing of skeletal muscle heavy microsomes with 0.5 M NaCl. Solubilization of washed membranes with 0.5% Triton X-100 released about half of this particulate nNOS, with the remainder found to be in an insoluble cytoskeletal pellet (Figure 1A). By contrast, eNOS, which is membrane-associated owing to N-terminal myristoylation, was quantitatively solubilized from these same preparations by Triton X-100 (Figure 1B).

The differential fractionation of nNOS and eNOS seen in Figure 1 suggests that unique determinants present in nNOS anchor this isoform to the skeletal muscle cytoskeleton.

To further investigate these findings, cofactor-binding domains of eNOS and nNOS were compared using a

schematic alignment. Results are seen in Figure 2 which shows that the extended N-terminus of nNOS contains a PDZ domain that is not present in eNOS and not required for enzyme normal activity.

Figure 2A shows schematic alignment of cofactor-binding domains of eNOS and nNOS, indicating N-terminal myristoylation (Myr) of eNOS and extended N-terminus of nNOS. The jagged line indicates the region deleted for the nNOSΔ1-226 mutant. CaM indicates calmodulin, FMN indicates flavin mononucleotide and FAD indicates flavin adenine dinucleotide regions.

As seen in Figure 2A, the amino acid sequence of nNOS contains a 230 amino acid N-terminal domain that is not present in eNOS. Beyond this extended N-terminus of nNOS, the two proteins share >60% sequence identity. Similar enzymatic activities of eNOS and nNOS suggest that the unique N-terminus of nNOS is not required for catalytic activity and has another function.

Figure 2B shows a schematic alignment of the PDZ domain of nNOS with syntrophins and a family of other cytoskeletal-associated proteins. T-SYN and SYN-1 indicate syntrophins, DLG indicates disks large, DSH indicates disheveled and PTP indicates protein-tyrosine phosphatase.

Rather than regulating catalytic activity, the N-terminal domain of nNOS instead seems to target nNOS to skeletal muscle sarcolemma. Within this domain, nNOS contains a 66 amino acid motif that bears homology to a heterogeneous family of signaling enzymes that share the property of being localized to specialized cell-cell junctions as seen in Figure 2B. Proteins containing this motif, which is named PDZ for a conserved tetrapeptide (Neuron, 9: 929-942 (1992)), include dlg-1, the product of the lethal discs large tumor suppressor gene that localizes to the undercoat of the septate junction in Drosophila; disheveled, a gene required for planar cell polarity in Drosophila; PSD-95, a brain-specific protein; ZO-1, a protein that localizes to tight junctions (zona occludens)

of epithelial and endothelial cells; and certain protein-tyrosine phosphatases such as PTP1E, which are localized at the junction between the plasma membrane and the cytoskeleton. Homology to syntrophins, a family of recently cloned dystrophin-binding proteins, which colocalize with nNOS beneath the sarcolemmal membrane of skeletal muscle, was observed.

In fact, as seen in Figure 2B, syntrophins are more closely related to nNOS in this domain than any other known gene, which suggests a dystrophin role in regulating the sarcolemmal localization of nNOS.

To analyze the role of the extended N-terminal region of nNOS, a deletion mutant nNOSΔ1-226, lacking the first 226 amino acids was constructed. Expression vectors containing full-length nNOS and nNOSΔ1-226 were transiently transfected into COS cells.

In Figures 2C and 2D, COS cells were transfected with 10 μg of the expression vector using a cytomegalovirus promoter to drive expression of either full-length nNOS (Fig. 2C) or the truncation mutant nNOSΔ1-226 (Fig. 2D). NOS activity was measured in cell homogenates 3 days following transfection in the presence of either 200 μM free calcium (full squares) or 2 mM EDTA (full circles). Kinetic constants (V_{max} and K_m) were calculated by Scatchard plot analysis. Data are means of triplicate determinations that varied by <10%. This experiment was replicated twice with similar results.

As seen in Figures 2C and 2D, kinetic characteristics of NOS activity for the nNOSΔ1-226 mutant was essentially indistinguishable from the full-length isoform. Both constructs displayed similar V_{max} and K_m for arginine as well as regulation by calcium/calmodulin.

Results of these experiments show that nNOS and eNOS associate with different cellular membrane fractions and that nNOS but not eNOS is present in cytoskeletal extracts from skeletal muscle.

Association of nNOS with Dystrophin

Based on the above findings of nNOS homology to syntrophins, dystrophin binding proteins, association of nNOS and dystrophin in skeletal muscle extracts was investigated.

Results of several studies of association of nNOS and dystrophin using affinity chromatography in skeletal muscle are seen in Figure 3. Affinity chromatography was performed according to Example 2. For Figures 3A-3C, protein homogenates (200 μg per lane) and aliquots of affinity eluates (150 μl per lane) were resolved on 7.5% (α-nNOS) or 6% (α-dystrophin) SDS gels and transferred to PVDF membranes, and immunoreactive bands were visualized by chemiluminescence.

A dystrophin-nNOS association complex was investigated by means of succinylated wheat germ agglutinin (sWGA) using sWGA-Sepharose affinity chromatography. This technique allows distinction between nNOS which, due to its lack of glycosylation sites does not bind to wheat germ column, while dystrophin binds to a glycoprotein complex.

To ensure specificity of this association, parallel experiments were conducted with *mdx* mice that specifically lack dystrophin owing to a nonsense mutation, but express nNOS at near-normal levels.

Quadriceps from wild-type and *mdx* mice were homogenized and solubilized in a buffer containing 0.2 M NaCl and 1% digitonin. Solubilized homogenates were applied to sWGA-Sepharose columns that were extensively washed with a buffer containing 0.5 M NaCl and 0.5% Triton X-100. Tightly bound proteins were affinity eluted with 0.3 M N-acetyl-D-glucosamine (NAG). Results are seen in Figure 3A.

Figure 3A shows dystrophin-associated glycoprotein complex and nNOS purified by sWGA chromatography from wild-type (WT), and *mdx* skeletal muscle.

As seen in Figure 3A, Western blotting indicates that total nNOS levels in crude extracts are similar in wild-

type (WT) and *mdx* muscle and reveals the presence of nNOS in NAG eluates from wild-type but not *mdx* tissue. nNOS coelutes with dystrophin on a sWGA affinity column in muscle homogenates from wild-type mice. nNOS does not adhere to an sWGA column in extracts from *mdx* mice.

Additionally, analogous experiments were conducted and association of dystrophin with nNOS was evaluated using a 2′,5′-ADP-agarose column, which tightly binds nNOS at its C-terminal NADPH-binding motif. Dystrophin has no nucleotide-binding site, and would not be expected to adhere to a 2′,5′-ADP column. To ensure specificity of a potential nNOS-dystrophin interaction, parallel purifications from skeletal muscle of nNOS knockout mice, which are devoid of full length nNOS protein yet express dystrophin at normal levels, were conducted. Salt-washed heavy microsomes from wild-type and full length nNOS knockout mouse quadriceps were solubilized in 1% digitonin and allowed to adhere to 2′,5′-ADP-agarose columns. The columns were extensively washed with buffers containing 0.5 M NaCl and 0.5% Triton X-100. Tightly bound proteins were eluted with buffers containing 20 mM NADPH. Results are seen in Figure 3B.

Figure 3B shows dystrophin-associated glycoprotein complex and nNOS purified by 2′,5′-ADP-agarose chromatography from wild-type (WT), *mdx*, and NOS knockout ($NOS^{-/-}$) skeletal muscle. As seen in Figure 3B, Western blotting for dystrophin reveals that dystrophin levels are equivalent in crude samples from wild-type (WT) and nNOS knockout ($NOS^{-/-}$) skeletal muscle. Dystrophin coelutes with NOS on a 2′,5′-ADP affinity column in muscle homogenates from wild-type but not for nNOS knockout mice.

To evaluate directly the binding of dystrophin-associated complexes to the N-terminal domain of nNOS, a protein containing glutathione S-transferase (GST) fused to the first 299 amino acids of nNOS was coupled to glutathione beads according to the method described in Example 3. The GST-nNOS(1-299) beads and control GST beads

were incubated with solubilized homogenates of mouse skeletal muscle. After extensive washing of the beads, bound proteins were eluted with sample buffer. Results are seen in Figure 3C.

In Figure 3C, glutathione-Sepharose beads bound to GST or GST-nNOS(1-299) were incubated with solubilized skeletal muscle membranes. After extensive washing, the beads were eluted with 0.2% SDS and proteins were separated by SDS-PAGE, and retention of dystrophin protein was analyzed by Western blotting. Western blotting indicated that GST-nNOS(1-299) beads but not control beads retained the dystrophin protein.

Other proteins which were concurrently evaluated, such as myosin, were retained in very small but equivalent amounts by both GST and GST-nNOS(1-299) beads indicating specificity of the association with dystrophin.

To quantitate enrichment of nNOS by sWGA chromatography, scale purifications from rat skeletal muscle tissue were conducted as seen in Figure 3D where L indicates load, F indicates flowthrough, W1 indicates 500 mM/NaCl wash, W2 indicates 500 mM NaCl and 0.5% Triton X-100 wash, and E indicates 0.3 M NAG eluate. Results are seen in Figure 3D.

Figure 3D depicts Western blotting for nNOS from equally loaded fractions (5 μg per lane) from sWGA chromatography shows large enrichment of nNOS in NAG eluate fractions.

To evaluate the retention of nNOS by sWGA, fractions from sWGA chromatography were reloaded onto SDS gels with 6-fold more total protein in load (L) and flowthrough (F) lanes (21 μg) than in wash (W1 and W2) and eluate (E) lanes (3.5 μg). Results are seen in Figure 3E.

In Figure 3E reloaded samples from the sWGA column show purification of nNOS and dystrophin, but not eNOS, by sWGA. Clearly, the majority of particulate nNOS adhered to the sWGA column as evidenced by the minimal amount of nNOS present in the flowthrough.

When the blot shown in Figure 3E was probed with antisera to dystrophin, it was found that essentially all the solubilized dystrophin adhered to the sWGA column and that dystrophin was enriched to a somewhat greater degree than nNOS by this procedure (~ 150-fold purification). The somewhat higher recovery of dystrophin appears to be due to slow dissociation of nNOS from the sWGA column due to its binding within the dystrophin complex under stringent conditions, as evidenced by some "bleeding" of nNOS with the 500 mM NaCl and 0.5% Triton X-100 wash steps (Figure 3E).

Additionally, this same blot was probed with an antibody to eNOS, which is similar to nNOS but lacks a PDZ domain. eNOS did not specifically adhere to the sWGA column, and only residual amounts of eNOS were found in NAG eluate fractions.

To determine whether the nNOS associates with dystrophin complexes eluting from sWGA, immunoprecipitation experiments were conducted. NAG eluate samples were incubated for 1 hour with a monoclonal antibody to α-dystrophin, and immunocomplexes were pelleted with anti-mouse immunoglobulin G (IgG) linked to protein A-Sepharose as described in Example 4. Results are seen in Figure 3F.

Figure 3F shows an immunoprecipitation of NAG eluate fractions with a monoclonal antibody to dystrophin (2.0 μg/ml; 12 nM) which precipitates nNOS (lane 1). Control experiments containing a monoclonal anti-Myc antibody (12 nM) obtained from BABCO, or lacking any primary antibody (lanes 2 and 3) fail to precipitate nNOS.

Western blot analysis seen in Figure 3F revealed potent immunoprecipitation of nNOS with α-dystrophin antibody. Control immunoprecipitations lacking the primary dystrophin antibody or containing an alternate monoclonal antibody anti-Myc did not precipitate detectable nNOS, demonstrating specificity of the interaction.

These studies show that nNOS coelutes with dystrophin on a sWGA or a 2′,5′-ADP affinity column in healthy muscle

homogenates but not in extracts from *mdx* or knockout mice and that dystrophin binds to the N-terminal domain of nNOS. Neuronal NOS from equally loaded fractions from sWGA chromatography shows large enrichment of nNOS in NAG eluate fractions and nNOS and dystrophin, but not eNOS, were purified by sWGA. This shows that a large fraction of nNOS actually binds to the dystrophin complex.

Immunoprecipitation of nNOS was found upon incubation with a monoclonal antibody to α-dystrophin, but not with other control antibodies.

These results clearly indicate association of nNOS with dystrophin or dystrophin-associated complex.

Displacement of nNOS from Sarcolemma

The absence of dystrophin was shown (J. Cell. Biol., 112: 135-148 (1991)) to lead to a dramatic reduction of other dystrophin-associated proteins, including α- and β-dystroglycan and syntrophins, in the sarcolemma of *mdx* mice and in patients with DMD (J. Biol. Chem., 266: 9161-9165 (1991)).

In order to determine whether the same is valid for nNOS, the distribution of nNOS in the *mdx* mice brain and skeletal muscle tissue was evaluated. Results are seen in Figure 4.

Figure 4 illustrates nNOS displacement from particulate fractions of the *mdx* skeletal muscle.

In Figure 4, subcellular fractions (S_1, S_2, S_3, and P) of mouse quadriceps from healthy wild-type (WT) and *mdx* mice (age matched at 7-8 weeks) were prepared as described in Figure 1 and nNOS and dystrophin were detected by Western blotting. Results are shown in Figures 4A and 4B and in Table 1.

Western blotting seen in Figure 4A shows that nNOS in the skeletal muscle of *mdx* mice is largely extracted from membranes with 100 mM NaCl (S_1) and is completely removed with 500 mM NaCl (S2). In wild-type mice, the majority of nNOS remains membrane-associated following the 500 mM NaCl wash. Of this remaining nNOS, approximately half is

removed by a 0.5% Triton X-100 extraction (S_3) and half is present in an insoluble pellet (P).

Figure 4B shows that dystrophin is enriched in detergent extract (S_3) and cytoskeletal pellet (P) fractions in wild-type mice (WT) and is completely absent from the *mdx* muscle.

As seen in Figures 4A and 4B, overall nNOS levels and enzyme activity were modestly decreased (~80% of control levels) in skeletal muscle from *mdx* mice. Subcellular analysis revealed that nNOS distributed with dystrophin in membrane-associated and cytoskeletal fractions from wild-type skeletal muscle. However, in preparations from *mdx* mice, nNOS was quantitatively solubilized from microsomal membranes washed with 0.5 M NaCl. No nNOS protein was detected in detergent extract or cytoskeletal fractions (Figure 4A).

Displacement of nNOS from skeletal muscle and brain tissue of WT and mdx mouse is seen in Table 1.

TABLE 1

NOS Activity in Extracts from Wild-Type and *mdx* Mouse-Tissues

NOS Activity	Wild Type[a]	*mdx*[b]
Skeletal muscle		
Soluble fraction	33.3	58.3
Particulate fraction	252	<0.5
Brain		
Soluble fraction	1020	1060
Particulate fraction	1080	1210

Particulate and soluble fractions of quadriceps skeletal muscle from wild-type and *mdx* mice were prepared and assayed for NOS activity as described in Example 1.

Data are means of triplicate determinations that varied by <10%.

[a]In counts per minute per milligram of protein.

As seen in Table 1, NOS catalytic activity was observed in soluble and particulate fractions in skeletal muscle and in brain in WT mouse tissue. In *mdx* tissue, nNOS was found in the soluble but not in the particulate

fraction. NOS activity in the soluble fraction of *mdx* skeletal muscle occurred at levels 75% greater than wild-type, but NOS activity was not detectable in the particulate fraction from *mdx* muscle. In the brain, NOS-specific activity was nearly equivalent in soluble and particulate fractions. This distribution was unchanged in the *mdx* brain, suggesting that proteins other than dystrophin anchor nNOS to neuronal membranes.

The above findings were confirmed with immunohistochemical findings of an absence of sarcolemmal nNOS in *mdx* skeletal muscle. Cryosections were prepared and immunostaining was performed according to Example 5. Results are seen in Figure 5.

Figure 5 shows immunofluorescent staining for nNOS in quadriceps of wild-type, *mdx*, dy, and nNOS knockout mice performed using an affinity-purified polyclonal antibody. Cryostat mice sections from wild-type, *mdx*, dy, and nNOS knockout mouse were stained under identical conditions using an affinity-purified nNOS antiserum and a FITC-linked secondary antibody.

Figures 5A and 5B show that nNOS immunostaining is present at the surface membranes of skeletal muscle fibers from wild-type (WT) mouse (A), but is absent from *mdx* mouse (B) skeletal muscle sarcolemma.

To determine whether this derangement of nNOS was specific for dystrophin abnormalities, the distribution of nNOS in dy mice, which have severe muscular dystrophy due to absence of an extracellular matrix protein, merosin, but have a normal distribution of dystrophin at the sarcolemma, was evaluated.

Figure 5C shows nNOS distribution in homozygous dystrophic dy mice displaying normal sarcolemmal nNOS labeling of intact fibers. Results show that nNOS is present normally at the sarcolemma of dy dystrophic mice (Figure 5C).

Figure 5D shows that skeletal muscle from NOS knockout ($NOS^{-/-}$) mice is entirely devoid of immunostaining.

The above results show that nNOS is selectively absent from sarcolemma of *mdx* skeletal muscle.

Using a polyclonal antiserum, nNOS immunofluorescence found in Figure 5 was restricted to the sarcolemma of a subset of WT skeletal muscle fibers. These fibers were previously noted to be fast twitch fibers (Nature, 372: 546-548 (1994)). However, nNOS immunoreactivity was absent from the sarcolemma of *mdx* muscle.

Several control experiments were conducted to ensure a specificity of the immunofluorescence. No specific labeling was present in muscle tissues incubated with preimmune serum or without primary antiserum. Most importantly, labeling was not present in transgenic knockout mice ($NOS^{-/-}$) containing a targeted mutation of the nNOSs gene (Figure 5D).

This study clearly shows that in dystrophic muscle tissue nNOS is displaced from sarcolemma but accumulates in cytosol.

Absence of nNOS from Skeletal Muscle Sarcolemma in Human Duchenne Muscular Dystrophy Patients

In follow-up studies described above, and to show that the same results are observed in animal models and controls, the localization of nNOS in healthy and DMD human muscle tissues was evaluated.

A total of 20 human tissues were prepared according to Example 8 and evaluated. These tissues included thirteen normal specimens and seven specimens from patients with DMD. Skeletal muscle cross sections were processed for immunofluorescence for nNOS, dystrophin, and spectrin. Results are seen in Figure 6.

Figure 6 are skeletal muscle cryosections of normal (N1) or DMD (D1) skeletal muscles immunostained with antibodies to dystrophin, nNOS, and spectrin.

Figure 6B shows representative muscle sections, labeled with antibodies, from two normal patients (N2 and N3) and two DMD cases (D2 and D3), to dystrophin and nNOS.

Figure 6C which represents control experiments using

two independently generated nNOS antisera shows similar staining of human tissues. No immunofluorescence was detected in the absence of primary (1°) antibody.

All normal specimens showed colocalization of nNOS, dystrophin, and spectrin beneath the sarcolemma of muscle fibers (Figures 6A and 6B), indicating that nNOS shares a similar distribution in both human and rodent skeletal muscle. In all seven biopsies from patients with DMD, the disruption of dystrophin resulted in absence of nNOS staining of sarcolemma (Figures 6A and 6B). Normal sarcolemma labeling for spectrin confirmed that this structural cytoskeleton was not disrupted in the DMD tissues (Figure 6A, spectrin). Two independently raised nNOS antibodies according to Example 3 yielded similar immunofluorescent staining patterns. No specific labeling was found in muscle sections incubated without primary antibody (Figure 6C).

Results seen in Figure 6 shows that nNOS is absent from sarcolemma of human DMD muscle fibers.

Absence of dystrophin in DMD results in disruption of the dystrophin-associated glycoprotein complex and in a dramatic reduction of overall levels of certain dystrophin-associated proteins in muscle. To evaluate total nNOS levels in skeletal muscle tissues from human DMD, Western blot analysis was conducted. Results are seen in Figure 7.

In Figure 7, skeletal muscle tissue homogenates from three cases of DMD and three normal muscle biopsies were resolved by SDS-PAGE as described in Example 1.

Immunoblot analysis seen in Figure 7A confirms that dystrophin is present in normal human muscle but is essentially absent from the human DMD muscle. Densitometric scanning of nNOS immunoreactive bands in equally loaded Western blots revealed ~75% decrease of nNOS in DMD tissues when compared to the normal human muscle. Immunoblotting for spectrin seen in Figure 7C confirmed that similar amounts of protein were loaded in all cases and that the structural cytoskeleton of these samples

remained intact.

The above series of studies demonstrates that both human and mouse skeletal muscle sarcolemmal nNOS is complexed with dystrophin. The dystrophin complex interacts with the N-terminus of nNOS, which contains a PDZ motif. In normal healthy muscle, nNOS is present in sarcolemma. In human DMD muscle and *mdx* mice, which lack dystrophin, nNOS is absent from the sarcolemma and accumulates in the cytosol. This derangement of nNOS is specific for dystrophin abnormalities, as nNOS disposition is unaffected in other muscular diseases.

The obtained results provide molecular evidence for a specific intracellular signaling molecule linked to the dystrophin-associated complex and suggest roles for NO in processes of neuro-muscular development and disease associated with this complex.

The PDZ domain is a protein motif that is present in a heterogeneous family of enzymes. The current invention investigated and discovered that deletion of the PDZ domain of nNOS does not alter NOS catalytic activity in transfected cells. A 299 amino acid fusion protein containing the PDZ domain in nNOS selectively retained dystrophin from skeletal muscle extracts, indicating that this domain is capable of interacting with the dystrophin-associated complex.

These results suggest potential roles for nNOS in neuromuscular signaling and disease associated with dystrophin. Association of nNOS with dystrophin completes the link between the extracellular matrix and intracellular signal-transducing enzymes. Aberrant translocation of nNOS from sarcolemma to cytosol in DMD and *mdx* muscle has implications for the pathogenesis of muscular dystrophy.

As nNOS has been unquestionably implicated in the DMD where the dystrophin is absent, and nNOS was shown to be displaced, the second series of studies was designed to investigate whether these findings would also be valid for other, not so severe, types of muscular dystrophies where

dystrophin is not completely absent but is mutated and to a certain degree dysfunctional.

Consequently, evaluation of nNOS distribution in a variety of muscle diseases and dystrophies was undertaken. In these follow-up studies it was found that nNOS occurs normally at the sarcolemma in human neurogenic muscle atrophy, central core disease, and severe childhood autosomal recessive muscular dystrophy but that it is, however, displaced from sarcolemma of Becker muscular dystrophy suggesting specificity of the defect of nNOS in DMD and BMD.

Selective Loss of Sarcolemmal NOS in Becker Muscular Dystrophy

Becker muscular dystrophy is a clinical variant of Duchenne muscular dystrophy. It differs from the more severe Duchenne dystrophy in that Becker muscular dystrophy patients do not lack dystrophin completely but their dystrophin is of abnormal molecular weight due to chronic mutation at Xp21. Becker muscular dystrophy patients, therefore, have reduced amounts of normal-sized dystrophin protein.

Becker muscular dystrophy is an X-linked disease due to mutations of the dystrophin gene. Mutations causing Becker's dystrophy are often in-frame deletions in the central rod-like domain of dystrophin that do not generally affect formation of the structural glycoprotein complex formed around dystrophin in the muscle.

In the studies described below, it was discovered that neuronal-type nitric oxide synthase (nNOS), an identified signaling component of the dystrophin complex, is uniquely absent from skeletal muscle sarcolemma in many human Becker's patients and in mouse models of Becker's dystrophy. This is in agreement with finding the same displacement of nNOS from sarcolemma in DMD patients.

A N-terminal PDZ domain of nNOS directly interacts with α1-syntrophin but not with other proteins in the dystrophin complex analyzed. However, nNOS does not

associate with α1-syntrophin on the sarcolemma in certain human Becker's dystrophy patients and in transgenic *mdx* mice expressing truncated dystrophin proteins. This suggests a macromolecular interaction of nNOS, α1-syntrophin and dystrophin *in vivo*, a conclusion supported by developmental studies in muscle. The data below indicate that proper assembly of the dystrophin complex is dependent upon the structure of the central rod-like domain and have implications for the design of dystrophin-containing vectors for gene therapy.

Sarcolemma nNOS Expression During Postnatal Muscle Development

This section describes studies performed to investigate sarcolemmal nNOS expression during postnatal muscle development.

The studies described above show that association of nNOS with the dystrophin complex is mediated by direct binding of the N-terminus of nNOS to the PDZ domain of α1-syntrophin.

In the current study, illustrated in Figure 8, localization of nNOS and other dystrophin associated proteins during postnatal development was investigated. In the postnatal day 3 (P3), day 7 (P7), day 12 (P12) and day 60 (P60) samples of rat quadriceps were obtained and investigated. Adjacent sections of postnatal rat quadriceps muscle were stained for dystrophin, nNOS, α-BGT, and utrophin and nearby sections were stained for α1-syntrophin and α-BGT. Staining was performed according to Nature Genetics, (1996).

As seen in Figure 8, dystrophin and nNOS stained extrajunctional sarcolemma at days P3 and P7 and both became concentrated at neuromuscular endplates at days P12 and P60. α1-syntrophin was present at extrajunctional sarcolemma and was enriched at neuromuscular endplates at all ages evaluated. Utrophin staining was restricted to neuromuscular endplates and was specifically enriched at neuromuscular endplates. By contrast at postnatal day 3

(P3) and P7, nNOS occurred only at extrajunctional sarcolemma and enrichment of nNOS at neuromuscular endplates did not become apparent until P12, which coincided with assembly of dystrophin complexes at endplates. Enrichment of α1-syntrophin at endplates of P3 and P7 muscle likely occurs by association with the dystrophin related protein, utrophin, which was enriched at endplates in all stages evaluated.

These studies indicate an apparent requirement of dystrophin for nNOS/α1-syntrophin colocalization at neuromuscular endplates during development and suggest that nNOS does not associate with utrophin-containing protein complexes.

Association of nNOS with Mutant Dystrophin Complexes in Transgenic Mice

In order to investigate whether nNOS association with the dystrophin complex correlates with the dystrophic disease phenotype, several transgenic mice models carrying either the full length dystrophin or truncated dystrophin were investigated.

In studies to investigate association of nNOS with mutant dystrophin complexes in transgenic mice, nNOS, dystrophin associated proteins (α-BGT) and α1-syntrophin expression were compared in skeletal muscle of wild type, *mdx* and various transgenic mice that express mutant forms of dystrophin, such as full-dys, Δ330, *mdx* ΔEXON 17-48, mini-dys or Dp71 mutant lines.

Results are seen in Figure 9 which shows localization of nNOS and α1-syntrophin in transgenic *mdx* mice. In Figure 9, cryosections from mouse quadriceps were immunofluorescently double labeled for either nNOS, α1-syntrophin and α-BGT.

As seen in Figure 9, immunofluorescent staining showed that nNOS in wild type mice was expressed at extrajunctional sarcolemma of a subset of fibers and was enriched at all neuromuscular endplates. nNOS was absent from junctional and extrajunctional sarcolemma in *mdx* mice.

nNOS staining in *mdx* transgenic mice expressing full length dystrophin (full-dys) or truncated dystrophin lacking the C-terminal 330 nucleotides (Δ330), resembled that of wild type mice. *mdx* mice expressing dystrophin lacking exons 17-48 (mini-dys) or lacking the C-terminal 71 kDa of dystrophin did not show nNOS staining at sarcolemma. These results corresponded to results observed in non-transgenic *mdx* mice.

α1-Syntrophin staining was observed at extra-junctional sarcolemma and was concentrated at neuromuscular endplates in wild type mice but was restricted to the endplates in *mdx* mouse. α1-Syntrophin expression was restored to sarcolemma in the four transgenic *mdx* mouse lines expressing different portions of the dystrophin gene.

These studies confirmed that α1-syntrophin was absent from extrajunctional sarcolemma of *mdx* mice, but remained at its neuromuscular endplates. nNOS was absent from both junctional and extrajunctional sarcolemma of *mdx* mice. By contrast nNOS was restored to the sarcolemma only by full length dystrophin and the Δ330 mutant. The absence of sarcolemmal nNOS was thus closely correlated with disease phenotype in these transgenics.

Biochemical confirmation of the above studies is illustrated in Figure 10.

Figure 10 shows subcellular distribution of nNOS in WT, *mdx*, and transgenic *mdx* mice. Mouse quadriceps skeletal muscle homogenates were sequentially extracted with buffers containing 100 mM NaCl (S1), 500 mM NaCl (S2), and 0.5% Triton X-100 (S3), leaving an insoluble cytoskeletal pellet (P).

Figure 10A is Western blotting indicating that nNOS was enriched in membrane associated and in pellet fractions in wild type mice (lanes 1) and in transgenic *mdx* mice expressing full length dystrophin (lanes 4). In *mdx* mice (lanes 2) and Dp71 transgenic *mdx* mice (lanes 3), nNOS was fully extracted by 500 mM NaCl and was absent from the membrane associated and cytoskeletal fractions.

In Figures 10B and 10C a similar fractionation was performed on muscle homogenates from wild type mouse (lanes 1), *mdx* mice expressing dystrophin lacking exons 17-48 (lanes 2), or *mdx* mice expressing full length dystrophin (lanes 3). In Figure 10B, nNOS was absent from membrane associated (S3) and cytoskeletal pellet (P) in *mdx* mice expressing the truncated dystrophin. In Figure 10C, reprobing the blot for α1-syntrophin had a generally similar fractionation in muscle from all three mice lines.

Biochemical studies illustrated in Figure 10 confirmed that nNOS did not associate with sarcolemma in *mdx* mice or transgenic *mdx* mice expressing either Dp71 or ΔE17-48. In wild type mice and *mdx* transgenic mice expressing full length dystrophin or Δ330, nNOS was enriched in membrane associated and cytoskeletal fractions, while in *mdx*, Dp71 and ΔE17-48 lines, nNOS was present only in soluble fractions of muscle. α1-syntrophin occurred in sarcolemmal fractions of all four lines of transgenic *mdx* mice evaluated.

Connections between nNOS and the presence of normal length or truncated dystrophin have been clearly established by these studies. When dystrophin was absent from the transgenic mouse phenotype, nNOS presence was only observed in soluble but not in sarcolemmal fractions.

Specific Binding of PDZ Domain of nNOS to α1-Syntrophin

Further studies were directed to answering the question whether the PDZ domain of nNOS binds to dystrophin or dystrophin-associated proteins.

As previously demonstrated, a sepharose column linked to the fusion protein selectively retained several components of the dystrophin complex from crude skeletal muscle extracts. To determine which components directly interact with nNOS *in vitro* the dystrophin complex was dissociated by briefly adjusting the pH of muscle extracts to 11 and then repeating the binding assays immediately after neutralizing the extracts. Previous studies (J.

Biol. Chem., 266: 9161-9165 (1991)) have demonstrated that this procedure reversibly dissociates dystrophin from associated proteins.

Consequently, the current studies were directed to investigation whether the PDZ domain of nNOS specifically binds to α1-syntrophin. In these studies, a consideration was given to results obtained in DMD studies showing that the N-terminal domain of nNOS is necessary and sufficient for interaction with the dystrophin complex. Therefore, interaction of dystrophin-associated proteins with a purified fusion protein containing the first 299 amino acids of nNOS was evaluated. Results are seen in Figure 11.

Figure 11A illustrates selective interaction of nNOS and α1-syntrophin using Western blotting. Crude solubilized membranes from mouse quadriceps were titrated with NaOH to pH 11, to dissociate the dystrophin complex, and were neutralized to pH 7.4 with 1 M TrisHCl. Native (native) and dissociated (dissoc) preparations were incubated with agarose beads linked to either GST or GST fused to the first 299 amino acids of nNOS (G-NOS). After extensive washing, beads were eluted with loading buffer and proteins resolved by SDS/PAGE.

Figure 11A shows that α1-syntrophin was selectively retained by G-NOS beads in both native and dissociated preparations. Reprobing the same blot with dystrophin as seen in Figure 11B, or α-sarcoglycan, as seen in Figure 11C, revealed that G-NOS beads retained these proteins from native protein preparations. However, following dissociation of the complex, neither dystrophin nor α-sarcoglycan bound to G-NOS. The 55 kD band observed in input lanes from α-sarcoglycan blot appears to be mouse IgG and was reactive with the secondary antibody used for western blotting. On the other hand, as seen in Figures 11A - 11C, α1-syntrophin continued to interact with the nNOS column but dystrophin and other components of the dystrophin complex were not retained.

Binding of nNOS to Skeletal Muscle Syntrophin through PDZ Interactions

In this study, nNOS binding to skeletal muscle syntrophin through PDZ interactions was determined.

Interaction of PDZ motifs found to be present in nNOS and PSD-95 protein in the brain, as described in Section II raised the possibility that the PDZ domain of syntrophin represents the binding site for association of nNOS with the dystrophin complex. Interaction between the PDZ containing domains of nNOS (amino acids 1-195) and α1-syntrophin (amino acids 59-166) was first evaluated by the yeast two hybrid system.

Results are seen in Table 2. As seen in Table 2 when co-transferred, these constructs reconstituted GAL4 transcriptional activity.

TABLE 2

Interaction Between nNOS and α1-Syntrophin

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	Colony Color	Growth
Lamin C (66-230)	α1-syntrophin (59-166)	White	-
p53 (72-390)	α1-syntrophin (59-166)	White	-
nNOS (1-195)	α1-syntrophin (59-166)	Lt. Blue	+

Yeast HF7c and Y187 cells were cotransformed with expression vectors encoding various GAL4-binding domain and GAL4 activation domain fusion proteins. Each transformation mixture was plated on two synthetic dextrose plates, one lacking tryptophan and leucine and the other lacking tryptophan, leucine and histidine. Growth was measured on histidine-deficient plates and color was measured by a β-galactosidase colorimetric filter assay according to Nature, 340: 245-6 (1989).

To biochemically evaluate this association, "pull-down" assays from skeletal muscle extracts using GST fused to the PDZ domain of nNOS (G-NOS) were conducted according to Example 12. Results are seen in Figure 21.

Figure 21 illustrates that α1-syntrophin binds to the N-terminal PDZ containing domain of nNOS.

Figure 21A shows results of "pull down" assays of solubilized muscle extracts from wild type or *mdx* mice using an nNOS (amino acids 1-299)-GST fusion protein which were done as described in Figure 19. Western blotting shows that α1-syntrophin from both wild type and *mdx* mice is selectively retained by the G-NOS column. Input was 20% protein.

Figures 21B and 21C show immunoprecipitations of solubilized muscle extracts with a polyclonal antibody to α1-syntrophin which show co-precipitation of (Figure 21B, lane 2) nNOS but not eNOS (Figure 21C, lane 2). Control experiments with non-immune serum show no precipitation of nNOS or eNOS (lanes 1). Bands at 55 kD represent immunoglobulin heavy chains.

Figures 21D and 21E are subcellular fractionation of nNOS which is altered in $nNOS^{\Delta/\Delta}$ mouse muscle. Homogenized muscle was sequentially extracted in buffer containing 100 mM NaCl (lanes 1), 500 mM NaCl (lanes 2) and 500 mM NaCl + 1% triton X-100 (lanes 3). These extracts were purified by 2'5'-ADP agarose chromatography and were resolved by SDS-PAGE. Figure 21D nNOS is present in both soluble and membrane associated fractions of wild type mice, while nNOS isoforms in $nNOS^{\Delta/\Delta}$ are restricted to the cytosol (Figure 21E).

α1-Syntrophin was selectively retained by the G-NOS column but did not associate with a control GST protein column (Figure 21A; lanes 1,3,4). Dystrophin is retained by a G-NOS column. The absence of dystrophin in *mdx* mice results in disruption of the dystrophin glycoprotein complex. Therefore, association of skeletal muscle syntrophin from *mdx* mouse with G-NOS was evaluated. Total α1-syntrophin levels were decreased ~50% in *mdx* muscle. Binding of α1-syntrophin to G-NOS was unaffected by the dystrophin deficiency (Figure 21A; lanes 2,5,6). To demonstrate association of nNOS with syntrophin in muscle extracts, we conducted immunoprecipitation experiments. A polyclonal antibody specific for α1-syntrophin selectively

precipitated nNOS from solubilized skeletal muscle microsomes (Figure 21B). No precipitation of nNOS occurred with non-immune antibody, and eNOS, was not specifically precipitated by the α1-syntrophin antibody (Figure 21C).

To determine the role of the PDZ domain for association of nNOS with sarcolemmal dystrophin complexes *in vivo* we evaluated the subcellular distribution of nNOS isoforms in nNOS Δ/Δ mouse. Skeletal muscle homogenates were sequentially extracted with physiologic saline, the 500 nM NaCl, and finally 0.5% triton X-100. Following 2'5' ADP agarose purification of the muscle extracts, Western blotting indicated that only the nNOSΦ form is expressed in muscle of nNOS Δ/Δ. nNOS in skeletal muscle runs as a doublet due to a 102 bp (34 amino acid) alternative splice near the middle of the gene. Subcellular fractionation indicated that nNOSγ was restricted to soluble fractions of skeletal muscle (Figure 21E), contrasting with the soluble/particulate distribution of full length nNOS in muscle (Figure 21D).

Protein overlay assays were performed to evaluate association of nNOS with individual domains of α1-syntrophin. The three known syntrophins (α1, β1, and β2) have a common domain structure consisting of two pleckstrin homology (PH) domains, a PDZ domain and a carboxy terminal domain unique to syntrophins (SU, syntrophin unique). Results are seen in Figure 22.

Figure 22 shows the interaction between the nNOS-GST (1-299) fusion protein and the four α1-syntrophin domain fusion proteins, PH1, PDZ, PH2 and SU. Notably, only PDZ associates with nNOS. Moreover, no prominent bands were detected when the domains were overlayed with GST alone. Syntrophin domain fusion proteins (containing the T7·Tag epitope) were also used to overlay GST and nNOS-GST (Figure 22). Only the PDZ domain of α1-syntrophin bound to nNOS and no binding was observed to GST alone.

Figure 22A is purified α1-syntrophin PH1 (25kDa, lanes 1), PDZ domain (15kDa; lanes 2), PH2 (18 kDa; lanes 3) and

SU domain (16 kDa; lanes 4) fusion proteins were resolved and overlayed with GST or nNOS (1-299)-GST. The position and relative amounts of the α1-syntrophin domain fusion proteins are indicated by immunoreactivity with a monoclonal antibody against the T7·Tag. Bound GST fusion proteins were detected by blotting with a monoclonal antibody to GST. Only nNOS-GST bound specifically to the PDZ domain of α1-syntrophin.

Figure 22B GST (lanes 1) and nNOS-GST (lanes 2) were separated and overlayed with α1-syntrophin domain fusion proteins (PH1, PDZ, or PH2) or blotted with a monoclonal antibody to GST. Bound syntrophin fusion proteins were detected with monoclonal antibody to T7·Tag. Of the syntrophin fusion proteins tested, only PDZ bound to nNOS; no binding to GST was detected.

Figure 22C shows co-localization of nNOS and α1-syntrophin immunofluorescence at skeletal muscle sarcolemma and neuromuscular junctions which was labeled by rhodamine α-bungarotoxin (BGT).

Figure 22D is a schematic model showing interaction of nNOS via with skeletal muscle α1-syntrophin (59K syn) connected to dystrophin dimer. The interaction of nNOS with syntrophin is via their respective PDZ domains. DG indicates dystroglycan.

Figure 22 shows the interaction between the nNOS-GST (1-299) fusion protein and the four α1-syntrophin domain fusion proteins, PH1, PDZ, PH2 and SU. Notably, only PDZ associates with nNOS. Moreover, no prominent bands were detected when the domains were overlayed with GST alone. Syntrophin domain fusion proteins (containing the T7·Tag epitope) were also used to overlay GST and nNOS-GST (Figure 22). Again, only the PDZ domain of α1-syntrophin bound to nNOS and no binding was observed to GST alone.

(1989).

Sarcolemmal nNOS Expression in Becker's Dystrophy

In this section, sarcolemmal expression of nNOS in Becker's dystrophy was investigated in order to clarify

whether mutations in the N-terminal or rod-like domains of dystrophin that cause BMD in humans are associated with altered localizations of nNOS. For this purpose, nNOS and α1-syntrophin expression in 12 BMD patients with molecularly defined deletions in the dystrophin gene were immunohistochemically evaluated. Immunohistochemical expression of nNOS, dystrophin and syntrophin was assessed blindly. Results are seen in Figure 12.

Figure 12 shows skeletal muscle cryosections from human biopsies from normal patients or from patients having DMD, BMD Δ EXON 45-47, BMD Δ EXON 10-42, and α-sarcoglycan disturbances were immunostained with antibodies to dystrophin, syntrophin, α-sarcoglycan, or nNOS. All four antibodies showed sarcolemmal staining in normal patients and essentially no sarcolemmal labeling in patients with Duchenne muscular dystrophy (DMD). In two patients with BMD, due to loss of exons 45-47 or exons 10-42 of dystrophin, immunofluorescent labeling for dystrophin, syntrophin, and α-sarcoglycan was detected at the membrane. By contrast, nNOS sarcolemmal staining for nNOS was undetectable in these two BMD patients. nNOS labeling was present in a patient with α-sarcoglycan deficiency.

As seen in Figure 12, nNOS is absent from skeletal muscle sarcolemma in certain patients with Becker muscular dystrophy.

Sarcolemmal expression of nNOS, dystrophin and syntrophin in Becker muscular dystrophy is illustrated in Table 3.

TABLE 3

Sarcolemmal Expression of nNOS, Dystrophin and Syntrophin in BMD

Diagnosis	Exons Deleted	Immunofluorescence at Sarcolemma		
		nNOS	Dystrophin	Syntrophin
Normal (S92-10906)	-	++++	++++	++++
Normal (94x-243)	-	++++	++++	++++
Mild BMD (S90-14162)	45-47	0	++++	++++
Mild BMD (1987)	52	+++	++++	++++
Mild BMD (KF22)	45-48	+	++++	++++
Int. BMD (91-1670)	3-6	+	+++	++++
Int. BMD (78-113)	1042	0	++++	++++
Int. BMD (S84-2812)	13-41	++	+++	++++
Int. BMD (590-536)	45	0	+	++
Sev. BMD (CS9004625)	8	0	+	+++
Sev. BMD (S88-2698)	3-7	0	+	+++
Sev. BMD (S90-14163)	45-47	0	+++	++++
Sev. BMD (S90-107002)	51-52	0	+	+++
α-sarcoglycanopathy 1	-	++++	++++	++++
α-sarcoglycanopathy 2	-	+++	+++	++++

Human muscle biopsies were labeled by immunofluorescence. Sarcolemmal labeling was blindly evaluated by three observers from 0 to ++++. Variation between observers never varied by more than one +, and in those cases, the majority score is reported here.

Table 3 shows that loss of sarcolemmal nNOS, but not α1-syntrophin expression was highly correlated with disease phenotype. Some of the patients, with mild to intermediate disease, showed reduced but detectable nNOS staining of sarcolemma. In several patients, loss of sarcolemmal nNOS occurred despite apparently normal assembly of other components of the dystrophin-associated glycoprotein complex seen in Figure 12. By contrast, nNOS expression was intact in two patients with α-sarcoglycan deficiency, suggesting that abnormalities of nNOS are not a consequence of muscular dystrophy, but are specific for dystrophin-linked disease.

A principal finding of this study is that assembly of nNOS into the dystrophin complex is dependent upon the structure of the N-terminal and rod-like domains of dystrophin. Understanding the mechanism for nNOS association with the dystrophin complex is particularly important because disruption of this interaction broadly correlates with disease phenotype in certain animal models of muscular dystrophy and in patients with BMD. Absence of sarcolemmal nNOS in *mdx* mice expressing a dystrophin mini-gene indicates a role for the rod-like domain of dystrophin.

Studies of nNOS expression in BMD patients demonstrate that non-overlapping deletions in the N-terminal or central domain of dystrophin disrupt recruitment of nNOS to the sarcolemma. These results indicate that a unique nNOS interaction domain may not be present in dystrophin, but that proper conformation is required for assembly of nNOS into the dystrophin complex.

Studies described above further indicate that direct interaction of nNOS with α1-syntrophin accounts for association of nNOS with the dystrophin complex. Three syntrophin genes have been identified and each contains two pleckstrin homology (PH) domains. The first PH domain is split by a PDZ motif, and the second PH domain is followed by a C-terminal region unique to the syntrophins (J. Biol. Chem., 270: 25859-25865, (1995)). Interaction of nNOS with α1-syntrophin is mediated by direct association of PDZ protein-binding interfaces near the N-terminus of nNOS and α1-syntrophin.

Studies described in this section are consistent with findings of the nNOS function in DMD and further demonstrate that α1-syntrophin, but not dystrophin, β-dystroglycan or α-sarcoglycan, directly binds to the PDZ domain of nNOS following dissociation of the dystrophin complex. NOS isoforms lacking a PDZ motif do not associate with the dystrophin complex further confirming that the PDZ domain of nNOS represents the relevant domain for interaction.

Neuronal NOS does not to interact with utrophin containing complexes. During muscle development, nNOS occurs only at sites co-localized for both α1-syntrophin and dystrophin at developing neuromuscular endplates. Similarly, utrophin complexes at neuromuscular endplates of *mdx* mice specifically lack nNOS. Taken together with biochemical studies showing a selective and direct interaction of nNOS with α1-syntrophin *in vitro*, sarcolemmal localization of nNOS seems to require a presence of both syntrophin and dystrophin.

Based on the cumulative evaluation of above described studies, it is evident that abnormality of nNOS expression is specific for dystrophin-related diseases. Immunohistochemical analysis for nNOS, therefore, is able to provide a reliable diagnostic test for detection of these diseases.

Additionally, abnormal expression of nNOS seems to play a role in the pathophysiology of BMD. Endogenous NO is involved in regulation of skeletal muscle development and its contractility. Disruption of these signaling pathways may contribute to abnormal muscle function and incomplete myofiber regeneration seen in muscular dystrophy. In *mdx* mice expressing Dp71 or dystrophin mini-gene, nNOS is the only known dystrophin-associated protein absent from the sarcolemma. In human BMD, loss of sarcolemmal nNOS expression broadly correlates with the severity of the disease.

Muscular Dystrophy Therapy

The above findings allow a design of genetic therapies for DMD, BMD and other muscular dystrophies.

A primary goal of muscular dystrophy therapy is restoration of fully functional dystrophin. The therapy, therefore, involves either the replacement of full length dystrophin or replacement of a fragment of dystrophin which assures binding of dystrophin with nNOS through syntrophin. The muscular dystrophy therapy thus involves replacement of dysfunctional or missing dystrophin with functional dystrophin or a functional fragment thereof.

All fragments of dystrophin which meet the requirement of binding with nNOS in sarcolemma through syntrophin are

intended to be within the scope of this invention.

Because of the large size of the dystrophin protein, the replacement of the whole dystrophin is difficult and therefore, although it is known that dystrophin is missing in DMD and is dysfunctional in BMD, so far such therapy has not been successful. With the discovery according to the invention that in the normal nondystrophic skeletal muscle dystrophin is colocalized with nNOS which binds to a PdZ motif of syntrophin, a dystrophin associated protein, and that in dystrophic muscles not only dystrophin but also nNOS is missing from sarcolemma of the skeletal muscle, it is clear that it is not necessary to replace the whole dystrophin but only the dystrophin fragments which are involved in formation of nNOS/sarcolemma/dystrophin complex. Consequently, the dystrophin fragments binding to syntrophin which in turn binds to nNOS, as seen in Figure 22D, in sarcolemma suffice for treatment of muscular dystrophic disorders.

Therefore, vectors used for production of proteins useful for treatment of muscular dystrophy to be used for gene therapy need to encode truncation mutants of dystrophin, that provide mutated gene replacement with dystrophin constructs that properly assemble nNOS which complete rescue of muscle function requires. Prior to their use in gene therapy, dystrophin constructs are analyzed to ensure that the y recruit nNOS to sarcolemma.

Any and all such constructs are intended to be included in a method for treatment of muscular dystrophies.

Gene therapy is performed according to methods known in the art for these purposes.

Muscular Dystrophy Diagnosis

Diagnosis of muscular dystrophy is based on detection of nNOS using immunohistochemical detection of nNOS, histologic analysis of nNOS or a combination of both.

NADPH diaphorase staining method, which is fast, easy and practical for routine use, is most preferred.

A diagnostic test is described in Example 22.

II. Diagnosis, Prophylaxis and Treatment of Stroke and Other Neurodegenerative Diseases

In addition to findings that nNOS plays a role in development and control of muscular dystrophies, it has now been discovered that in the brain certain proteins, particularly postsynaptic density proteins PSD-95 and PSD-93 localized at synaptic junctions, also bind to PDZ domain of nNOS. In this way they may become important for management, therapy and diagnosis of stroke and other neurodegenerative diseases. Binding protein PSD-93 is novel and has never before been disclosed or described. Finding that PSD-95 and PSD-93 proteins bind to the PDZ domain of nNOS in the brain is also novel and was never before disclosed or described.

Use of nNOS Binding Proteins in Diagnosis, Prophylaxis and Therapy of Stroke and Other Neurodegenerative Diseases

Neuronal NOS is concentrated at synaptic junctions in the brain where the N-terminus domain of nNOS, which contains a PDZ protein motif, interacts both in vivo and in vitro with the second PDZ motif present in postsynaptic density-95 or -93 proteins. The second PDZ domain mediates binding of nNOS to the N-methyl-D-asparagine (NMDA) receptor located at the synapse through the first and/or the third PDZ domains of the PSD-95 or PSD-93.

Two binding proteins were found and identified to be involved in the binding of the nNOS to the NMDA receptor. They were cloned and expressed, and fusion proteins were prepared. Additionally, both these proteins were found to colocalize with nNOS. Additionally, small peptides corresponding to the carboxy terminal nine amino acids of N-methyl-D-aspartate (NMDA) type glutamate receptor were identified as inhibitors of binding of the nNOS binding proteins with the NMDA.

The invention also describes a method of use of nitric oxide, neuronal nitric oxide synthase, its binding proteins and their inhibitors, for diagnosis, prophylaxis and treatment of stroke and other neurodegenerative diseases, such as Huntington disease, amyotropic lateral sclerosis, Alzheimer

disease, etc. as well as a diagnostic assay for detection of absence of binding proteins or nNOS.

Finally, the invention concerns a binding assay for monitoring of binding of nNOS binding proteins with nNOS and appropriate synaptic receptors useful for development of compounds for treatment of neurodegenerative diseases.

Interaction of NOS with the Synaptic Density Proteins PSD-95 and PSD-93

Nitric oxide plays important physiological role in neurotransmission and synaptic modulation in central nervous tissue. Endogenous neuronal NO participates in development of some forms of neurotoxic injury, including stroke and other neurodegenerative processes. Functionally, NO mediates certain aspects of synaptic plasticity and neurotoxicity associated with NMDA receptors, but it does not play a major role in other pathways. In the brain, nNOS activity is selectively activated by a calcium influx through the NMDA receptor. Both nNOS and NMDA receptors are concentrated at synaptic junctions in the brain. Consequently, understanding of NO neurotoxicity requires identification of the functional connection of nitric oxide synthetic enzyme nNOS with NMDA receptors. For interacting connection of nNOS with NMDA receptor, a linker able to bind these two entities together is necessary. Two proteins, PSD-95 and PSD-93, have been identified as possible binding linkers.

The N-terminus domain of nNOS, which contains a PDZ protein motif was shown to interact with the second PDZ motif in postsynaptic density-95 protein (PSD-95) and a related novel PSD-93 protein.

Synaptic organization of nNOS at a synaptic junction is schematically shown in Figure 13 which is a molecular model of nNOS/N-methyl-D-aspartate receptor (NMDAR) binding mediated by PSD-95 or PSD-93 proteins. These proteins are known to associate with the glutamate type receptors to which NMDA receptor belongs.

As seen in Figure 13, endogenous NO is derived from L-arginine by nNOS. nNOS is concentrated at synaptic junction

in close vicinity of NMDAR. In the brain, neuronal NO synthase (nNOS) activity is selectively regulated through calcium influx controlled by NMDA receptors. Two post-synaptic density proteins PSD-95 and PSD-93, also located in the vicinity of the synaptic junction, are physically able to associate with nNOS through their respective PDZ domains. This shows that NMDA and nNOS are able interact with nearby binding sites in the second PDZ domain of PSD-95.

Nitric Oxide Synthase Binding Proteins

In the brain nNOS is thus functionally coupled to N-methyl-D-aspartate receptors. The N-terminal domain of nNOS is unique to the neuronal isoform and contains a PDZ motif of about 100 amino acids that is found in a diverse group of cytoskeletal proteins and enzymes. Because this domain was shown to mediate association of nNOS with the dystrophin complex, as described in section I, attempts were made to identify interacting proteins in the brain to perform the same function.

This invention demonstrates that nNOS is enriched at synaptic junctions in the brain owing to association of nNOS with the postsynaptic density proteins, specifically with the two proteins identified as PSD-95 and PSD-93. The PSD-95 protein clusters NMDA receptors at central nervous system synapses. PSD-95 and PSD-93 proteins therefore act as interacting proteins between nNOS and NMDA receptors.

Postsynaptic density protein PSD-95 was originally identified as an abundant detergent-insoluble component of brain postsynaptic density. Subcellular and electron micrographic studies have determined that PSD-95 is localized at both pre- and post-synaptic membrane and has a similar distribution to nNOS. PSD-95 contains three PDZ repeats, a SH3 domain and a region homologous to guanylate kinase (Neuron, 9: 929-942 (1992)). As seen in Figure 13, the second domain of PSD-95 provides the connecting link between nNOS and NMDA receptor.

Schematic representation of PSD-95 and PSD-93 proteins is seen in Figure 14. As seen in Figure 14, cloning and

sequencing of the PSD-95 related gene derived a protein PSD-93 of 93 kD, that has the same domain structure as PSD-95 and shares with PSD-95 about 60% amino acid identity. The nucleotide sequence of PSD-93 (SEQ ID NO: 1:) has been deposited in GenBank. The PSD-93 nucleic acid sequence contains 2963 base pairs. The amino acid sequence of PSD-93 are depicted by SEQ ID NOs: 2-6 containing cumulatively 987 amino acids. The amino acid sequence of PSD-95 is seen in Figure 14 and is identified as SEQ ID NOs: 15-19.

This invention thus identifies for the first time two brain proteins that physically associate with the enzyme neuronal nitric oxide synthase (nNOS). One of these nNOS binding proteins, postsynaptic density-95 (PSD-95) was previously cloned. The other, PSD-93 nNOS binding protein, is a novel protein whose cloning, sequencing and functional expression has never before been disclosed.

These proteins have now been shown to be involved in development and progression of stroke and other neurodegenerative diseases. Their inhibition, inhibition of their binding domain PDZ (second domain) or the inhibition of their binding with nNOS are all important for treatment, prophylaxis, detection and diagnosis of stroke and neurodegenerative diseases.

Colocalization of nNOS and PDS-95 in Adult and Developing Neurons

In order to determine whether the association of nNOS with PSD-95 and PSD-93 is physiologically relevant, colocalization of nNOS and PSD-95 or PSD-93 in adult and developing neurons was investigated. Their co-expression in neurons was studied using *in situ* hybridization.

The yeast two-hybrid system was used to identify interacting proteins. Screening a brain library demonstrated that the PDZ containing domain of nNOS binds to PDZ repeats present in PSD-95 and in a novel related protein, PSD-93. PSD-95 was found to be co-expressed with nNOS in several neuronal populations in the developing and mature nervous system, and a specific PSD-95/nNOS interaction was detected

in transfected cell lines and solubilized cerebellar membranes. On the other hand, residual catalytically active nNOS isoforms identified in $nNOS^{\Delta/\Delta}$ mice, that specifically lack a PDZ motif, did not interact with PSD-95. These data demonstrate a physiological role for PDZ domain interactions in organizing proteins at synaptic membranes. Interaction of nNOS and PSD-95 or PSD-93 via their PDZ domains therefore mediates synaptic-association of nNOS, with the NMDA receptor and may play a more general role in formation of macromolecular signaling complexes.

The study was based on the premise that if the association of nNOS with PSD-95 identified in yeast is physiologically relevant, the two proteins must be co-expressed in neurons. Results are seen in Figure 15.

Figure 15 shows expression of PSD-93, PSD-95 and nNOS in rat brain and in E15 embryo. *In situ* hybridization was used to localize transcripts for PSD-93 (inset A), PSD-95 (inset B), nNOS (inset C) or sense control (inset D) in adjacent cryosections prepared according to Example 5.

Figure 15A shows that in an adult rat brain, PSD-95 was observed only in neurons and was co-expressed with nNOS in certain neurons in hypothalamus, hippocampus and cerebellum. PSD-93 also appeared to be neuron specific, but had a more restricted distribution than did PSD-95.

Figure 15B shows that in the cerebellum, PSD-95 and nNOS were co-expressed in cerebellar granule cells in the granular layer (G) and basket cells (B) in the molecular layer. By contrast, PSD-93 was restricted to Purkinje neurons (P) of the cerebellum, which lack nNOS or PSD-95. Double labeling with NADPH diaphorase and *in situ* hybridization identified the PSD-95 and nNOS expressing cells in molecular layer as basket cells.

Figure 15C shows that in E15 embryo, PSD-95 was found ubiquitously expressed in differentiated central neurons, but not in neuronal precursors. PSD-95 was co-expressed with nNOS in the cerebral cortical plate (CP), dorsal root ganglia (DRG) and neurons of the olfactory epithelium (OE). PSD-93 was

specifically co-expressed in neurons of the spinal cord (SC), DRG and trigeminal nerve (V). PSD-93 was specifically co-expressed with nNOS in secretory cells of the submandibular gland (SG) and in (Figure 15D) chromaffin cells of the developing adrenal gland, which lack PSD-95. The signal in liver (L) seen in all samples including control represents non-specific hybridization to an aberrant fold in the tissue. K identifies the kidney. A identifies the adrenal gland.

In situ hybridization in a rat brain (Figures 15A and 15B) demonstrated co-expression of nNOS and PSD-95 transcripts in several neuronal populations, particularly in cerebellar granule and basket cells, which have previously been shown to express high densities of nNOS and PSD-95 proteins. On the other hand, PSD-93 occurred at highest densities in cerebellar Purkinje neurons, complimentary to the distribution to nNOS and PSD-95.

When expression of nNOS with PSD-95/PSD-93 during embryonic development was compared, it was found that nNOS-containing cells in embryonic day 15 (E15) were differentially co-expressed with either PSD-95 or PSD-93 (Figures 15C and 15D). As previously reported, transient NOS neurons were detected in developing cerebral cortical plate, olfactory epithelium, and sensory ganglia. In all of these neuronal groups PSD-95 mRNA was found. nNOS was also developmentally expressed in certain non-neuronal cells including chromaffin cells of the adrenal gland and secretory cells of the submandibular gland. PSD-93 mRNA and nNOS mRNA were co-expressed in these glands, while PSD-95, which is neuron specific, was absent.

Co-localization of nNOS and the PSD-95 protein was additionally evaluated by immunohistochemical staining of adjacent sections from an E19 rat. Results are seen in Figure 16.

Figure 16 shows that PSD-95 co-localizes with nNOS in developing neurons. Immunohistochemical staining of adjacent sagittal sections of an E19 rat fetus indicates that PSD-95 (Figure 16A and 16C) and nNOS (Figures 16B and 16D) are co-

localized in primary olfactory epithelium (OE) and in nerve processes projecting to the olfactory bulb (OB) (Magnification in Figures 16A and 16B is 50X; in Figures 16C and 16D is 400X). In the intestine (Int) of an E19 rat, as seen in Figures 16E and 16G, PSD-95 and, as seen in Figures 16F and 16H, nNOS are co-localized in myenteric neurons (MN). (Magnification 50X, and 200X, respectively.)

In an E19 rat cerebral cortex, as seen in Figure 16I, PSD-95 and nNOS (Figure 16J) are also co-localized. Both proteins are most concentrated in neuronal processes of the intermediate zone (IZ) and cell bodies of the cortical plate (CP), while the ventricular zone (VZ) is devoid of staining (Magnification 100X).

Within the olfactory system, both PSD-95 and nNOS were enriched in dendritic specializations in olfactory cilia and in axonal processes projecting to the olfactory bulb, which itself does not contain either nNOS or PSD-95, as seen in Figure 16A-D.

nNOS also occurs in fetal myenteric neurons and its absence is associated with hypertrophic pyloric stenosis. Immunohistochemical analysis revealed a co-localization of nNOS with PSD-95 in myenteric neurons (Figures 16 E-H). nNOS and PSD-95 were similarly co-localized in embryonic cerebral cortex. Staining for both proteins was enriched in the intermediate zone and in developing cortical plate, while lesser staining was found in the subplate region. The ventricular zone was devoid of staining (Figures 16I and 16).

These results show that both PSD-95 and PSD-93 are co-localized with nNOS in neuronal tissue and therefore must have a physiological importance connected with this tissue in conjunction with nNOS.

The Binding of nNOS Containing PDZ Domain to Similar Motifs of PSD-95 and PSD-93

In this study, binding of the PDZ containing domain of nNOS to similar motifs in PSD-95 and to a related protein, PSD-93 was investigated.

To determine which domain or domains of PSD-95 bind to

nNOS, yeast constructs encoding appropriate fragments of PSD-95 were fused to the GAL4 activation domain. Constructs encoding the second PDZ motif of PSD-95 interacted with nNOS while those lacking this region were inactive. Results are seen in Table 4.

Table 4

Interactions Between nNOS and PSD-95 or PSD-93

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	Colony Color	Growth
nNOS (amino acids 1-195)	SV 40 (amino acids 84-708)	White	-
p53 *72-390)	PSD-95, PDZ1-3 (20-364)	White	-
Lamin C (66-230)	PSD-95, PDZ1-3 (20-364)	White	-
nNOS (1-195)	PSD-95, PDZ1-3 (20-364)	Blue	+
nNOS (1-195)	PSD-95, PDZ1-2 (20-294)	Blue	+
nNOS (1-195)	PSD-95, PDZ2-3 (138-364)	Blue	+
nNOS (1-195)	PSD-95, PDZ1 (20-144)	White	-
nNOS (1-195)	PSD-95, PDZ2 (138-294)	Blue	+
nNOS (1-195)	PSD-95, PDZ3 (291-364)	White	-
p53 (72-390)	PSD-93 (116-421)	White	-
Lamin C (66-230)	PSD-93 (116-421)	White	-
nNOS (1-195)	PSD-93 (116-421)	Blue	+
p53 (72-390)	SV 40 (84-708)	Blue	+

When the PDZ domain of nNOS (amino acids 1-195) was fused to the DNA binding domain of GAL4 and screened by a human brain library for interacting proteins using the yeast two-hybrid system according to Nature, 340: 245-246 (1989) (Clonetech), two families of interacting clones were identified from a screen of 10^6 plasmids as seen in Table 4.

One family represented isolates encoding the PDZ motifs of PSD-95, and the other family encoded PDZ repeats of a related novel gene product, protein PSD-93. These findings show that PSD-95 and PSD-93 are related, both structurally, as seen from Figure 14, and also functionally, as their respective clones interacted with nNOS.

Interaction of nNOS with the PDZ Motif of PSD-95

As seen in Figure 13, the second PDZ domain of PSD-95 provides a binding link between the nNOS and NMDA receptor. In this study, confirmation of nNOS interaction with the second PDZ domain of PSD-95 was investigated. To evaluate formation of a nNOS/PDS-95 complex in the brain, immunoprecipitation studies were conducted. Results are seen

in Figure 17.

Figure 17 shows co-immunoprecipitation of nNOS and PSD-95. In Figure 17A, COS cells were transfected with an expression construct PSD-myc, encoding amino acids 1-386 of PSD-95 with a 10 amino acid c-myc epitope tag alone (lanes 1) or were co-transfected with PSD-myc and nNOS (lanes 2). Cell homogenates were immunoprecipitated with nNOS and probed with a monoclonal antibody to c-myc. Input was 5% protein loaded onto columns.

In Figure 17B solubilized cerebellar membranes were immunoprecipitated with antibody to PSD-95 (lanes 2) or a non-immune serum (lanes 1). Western blotting shows specific co-immunoprecipitation of nNOS but not eNOS with PSD-95.

Figure 17C shows identical immunoprecipitations from cerebellar cytosol, which lacks PSD-95 but contains high concentrations of nNOS. Figure 17C shows that the PSD-95 antibody (lane 2) does not directly interact with nNOS. The eNOS blot and the nNOS blot from cerebellar cytosol were intentionally overexposed, but failed to show specific immunoprecipitated bands.

Figure 17D shows affinity chromatography which demonstrates that nNOS is selectively retained by an immobilized PSD-95 protein fragment (amino acids 1-386) fused to GST. eNOS is not retained by the PSD column. Solubilized brain extracts were incubated with G-PSD or control GST beads, columns were washed with a buffer containing 0.5 M NaCl and 1% triton X-100, and eluted with SDS. Bound proteins were detected by Western blotting. Input was 10% protein.

Figure 17E shows that NMDA receptor 2B carboxy terminal peptide displaces nNOS and $K_v1.4$ from PSD-95. "Pull-down" assays from brain were conducted as above containing 0 (lanes 6, 7), 10 μM (lanes 4,5) or 30 μM (lanes 2,3) NMDA receptor peptide or 200 μM control peptide (lanes 8,9). Input was 10% protein.

Figure 17 shows and confirms that nNOS binds to PSD-95 through PDZ motif interactions. A nNOS-PSD-95 complex was immunoprecipitated from COS cells co-transfected with

expression vectors for nNOS and the PDZ repeats of PSD-95, indicating that this interaction occurs in a cellular environment (Figure 17A). Though only a small fraction of PSD-95 can be solubilized from brain densities with non-denaturing detergents, a nNOS/PDS-95 complex was specifically immunoprecipitated from cerebellum (Figures 17B and 17C), where both proteins are co-expressed at high levels.

To biochemically evaluate the interactions of nNOS and PSD-95, a fusion protein linking glutathione-S-transferase (GST) to the first 386 amino acids of PSD-95 (G-PSD) was generated. Solubilized brain extracts were incubated with glutathione beads linked to GST or G-PSD. Following extensive washing with a buffer containing 350mM NaCl and 0.5% triton X-100, bound proteins were eluted with a loading buffer. Western blotting indicated selective retention of nNOS to G-PSD beads but not of eNOS, which is 60% identical to nNOS but lacks a PDZ motif. These results indicate specificity of the interaction with nNOS (Figure 17D).

Because the second PDZ motif of PSD-95 binds to both nNOS and to tSXV, a protein motif of the NMDA receptor, containing ion channels, the investigation was undertaken to determine whether these binding sites are independent or overlapping. Studies described above demonstrated that a peptide corresponding to the carboxy terminal nine amino acids of NMDA receptor type 2B blocks interaction of NMDA receptor with PSD-95 (Science, 269: 1737-1740 (1995)). As seen in Figure 17E, this NMDA receptor peptide potently blocks association of nNOS with PSD-95. Half maximal inhibition of binding was achieved at <10 μM NMDA receptor peptide, while control peptides were inactive at 200 μM. As a control K_v 1.4, a voltage-dependent K^+ channel containing a tSXV sequence, was found to be displaced from PSD-95 by similar concentrations of NMDA receptor peptide.

The importance of the second PDZ domain of PSD-95 or PSD-93 for nNOS/PSD-95 binding was confirmed when the second PDZ domain of PSD-93 expressed as a GST-fusion protein was found to bind to nNOS in a manner competitive with the NMDA receptor

peptide.

Presence of Catalytically Active nNOS Isoforms Lacking a PDZ Motif in nNOS Δ/Δ Mice

To confirm that a PDZ motif is responsible for interaction of nNOS to PSD-95 or PSD-93, catalytically active nNOS isoforms lacking a PDZ motif observed in $nNOS^{\Delta/\Delta}$ mice were investigated.

Mice carrying a targeted disruption of exon 2 of nNOS express residual nNOS isoforms specifically lacking the PDZ domain. Thus these mice are extremely suitable for investigation whether nNOS isoform lacking the PDZ motif will bind to PSD-95 or PSD-93.

Neuronal $NOS^{\Delta/\Delta}$ mice were generated by deleting the first translated exon, which is exon 2, of nNOS in both mice and humans which encodes the PDZ motif. Results are shown in Figure 18.

Figure 18 illustrates that exons 1 and 2 of nNOS are alternatively spliced.

Figure 18A is Northern blot analysis of brain mRNA from wild type (WT) and $nNOS^{\Delta/\Delta}$ mice hybridized with a full length nNOS cDNA probe. A broad band of 10.5 kb is recognized in the wild type mouse brain and weaker bands of 11 and 9.5 kb are recognized in $nNOS^{\Delta/\Delta}$ mice.

Figures 18B and 18C show RT-PCR analysis of 5′ splicing of nNOS gene. For this study, cDNA was amplified with primers 1 and 2 (lanes 1,2).

Figure 18B is ethidium bromide staining which shows a band of 1 kb amplified from $nNOS^{\Delta/\Delta}$ and a band of 2.2 kb from wild type.

Figure 18C is Southern hybridization with a full length nNOS probe showing hybridization to the ethidium stained bands. A weaker band of 1 kb in amplifications of wild type cDNA is also detected by hybridization (lane 2). A similar analysis using primers 3 and 4 confirms that exon 2 sequences are only detected in wild type cDNA (lanes 3,4).

Figure 19 shows that catalytically active nNOS isoforms lacking exon 2 are expressed in the brain of $nNOS^{\Delta/\Delta}$ mice.

Figure 19A is Western blotting of crude (lanes 1,2) and 2' 5'-ADP affinity purified (lanes 3,4) brain extracts, indicating that the major nNOS band in wild type brain migrates at 160 kD (lanes 1,3) while in $nNOS^{\Delta/\Delta}$, co-purifying bands of 125 and 136 kD (lanes 2,4) are observed. Partial tryptic digestion of 2'5-ADP agarose-purified proteins reveals a similar proteolytic "fingerprint" from wild type (lane 5) and $nNOS^{\Delta/\Delta}$ (lane 6). 20 fold more protein was loaded from $nNOS^{\Delta/\Delta}$ (lanes 2,4,6) than from wild type samples (lanes 1,3,5).

Figure 19B shows cDNA clones encoding nNOS, nNOSβ (5'a spliced to exon 3) or nNOSγ (5'b spliced to exon 3) were transfected (trx) into COS cells and protein extracts were resolved by SDS/PAGE. Full length NOS (nNOS-trx) comigrates at 160 kD with the major product from the wild type brain (lanes 1,2). Transfection of nNOSβ and nNOSγ yields proteins of 136 and 125 kD respectively that comigrate with immunoreactive bands from $nNOS^{\Delta/\Delta}$ (lanes 3,4,5).

Figure 19C shows NOS catalytic activity of nNOS isoforms. COS cells were transfected with 10 μg of expression vector encoding full length nNOS, nNOSβ, or nNOSγ. NOS activity was measured in cell homogenates three days following transfection in the presence of 200 μM free calcium. This experiment was replicated twice with similar results.

Figure 19D shows that nNOS isoforms are discretely expressed in the $nNOS^{\Delta/\Delta}$ brain. Highest densities of nNOS in wild type (10 μg/lane) are found in the cerebellum (Cb). In $nNOS^{\Delta/\Delta}$ (100 μg/lane) highest levels of nNOS isoforms are found in striatum (St) and hippocampus (Hi), lower amounts are found in brainstem (Bs) and cerebral cortex (Cx) while the cerebellum is devoid of nNOS isoforms in $nNOS^{\Delta/\Delta}$. Figure 19E eNOS is homogeneously distributed in forebrain (Fb), cerebellum (Cb) as well as the peripheral tissues liver (Li), lung (Lu) and kidney (Ki). All lanes in Figure 19C were loaded with 100 μg of solubilized membrane extract.

Transfection of the 5'a containing construct generated a prominent immunoreactive protein band of 136 kD that comigrated with nNOSβ from $nNOS^{\Delta/\Delta}$ brain (Figure 19B; lanes

3,4). Transfection of the 5'b containing construct yielded a nNOS band of 125 kD (Figure 19B, lane 5). Catalytic assays indicated that the 136 kD nNOSβ form had activity ~80% that of full length nNOS under these transfection conditions (Figure 19C). Enzyme activity was fully dependent on calcium/calmodulin and the K_m for arginine was similar to that of full-length nNOS from the brain. By contrast, the activity of the 125 kD nNOSγ was ~3% that of nNOS.

NOS activity in the wild type brain is highest in the cerebellum. By contrast, NOS activity in nNOS$^{\Delta/\Delta}$ is highest in the striatum and lowest in the cerebellum. The regional distribution of residual nNOS isoforms in nNOS$^{\Delta/\Delta}$ brain extracts paralleled the pattern of residual nNOS activity previously reported in Cell, 75: 1273-86 (1993).

Absence of a PDZ Motif Prevents Association of nNOS Isoforms with PSD-95 or with Brain Membranes

In this study, association of nNOS isoforms lacking a PDZ motif with PSD-95 or brain membranes was investigated.

nNOS$^{\Delta/\Delta}$ mice express nNOS isoforms specifically lacking the PDZ motif were used as an important tool to determine the functions for this domain *in vivo*. Association of residual nNOS isoforms with PSD-95 was investigated. nNOS proteins purified from wild type and nNOS$^{\Delta/\Delta}$ mouse forebrain were subjected to pull-down assays as described above. Results are seen in Figure 20.

Figure 20 shows that nNOS isoforms lacking the PDZ motif do not bind to PSD-95 or to brain membranes.

In Figure 20A partially purified nNOS protein from wild type (WT) or nNOS$^{\Delta/\Delta}$ brains were analyzed by PS "pull-down" assay. Full length nNOS bi ds to PSD-95 while the residual isoforms lacking the PDZ mot_f do not. Input was 20% protein.

Figure B shows that residual nNOS isoforms are restricted to cytosol of nNOS$^{\Delta/\Delta}$. Brain homogenates, extracted with 100 mM NaCl (lanes 1), 1 M KCl + 1% triton X-100 (lanes 2) or insoluble pellet (lanes 3), from wild type (20 μg/lane) or nNOS$^{\Delta/\Delta}$ (200 μg/lane) were probed by Western blotting.

Only full length nNOS protein containing the PDZ motif was retained by G-PSD beads; the alternatively spliced forms in $nNOS^{\Delta/\Delta}$ did not adhere to G-PSD (Figure 20A). The distribution of nNOS in wild type and $nNOS^{\Delta/\Delta}$ mice by subcellular fractionation was compared. Brain homogenates were first extracted with physiological saline, then with buffer containing 1M KCl and 1% triton X-100, leaving a cytoskeletal pellet. nNOS in wild type brain was present in all fractions while residual nNOS isoforms in $nNOS^{\Delta/\Delta}$ occurred only in the first soluble fraction (Figure 20B).

The studies represented in Section II show that in the brain nNOS is functionally coupled to N-methyl-D-aspartate receptors through the interaction with binding proteins. The N-terminal domain of nNOS is unique to the neuronal isoform and contains a PDZ motif of about 100 amino acids. nNOS is enriched at synaptic junctions in the brain owing to association of nNOS with the postsynaptic density proteins PSD-95 and PSD-93 which act as interacting proteins between nNOS and NMDA receptors. The interaction of nNOS and PSD-95 or PSD-93 via their respective PDZ domains mediates synaptic-association of nNOS with the NMDA receptor. Association of nNOS with PSD-95 is physiologically relevant as the two proteins are co-expressed in neurons. nNOS PDZ domain interacts with the second PDZ domain of the PSD-95 or PSD-93 and the PDZ domain of nNOS is important for its interaction with the NMDA receptor. When this domain is missing, the interaction between nNOS and PSD-95 is also missing. Absence of a PDZ domain thus prevents the binding of nNOS/PSD-95 or PSD-93 proteins and nNOS interaction with the NMDA receptor.

Neuronal NOS and its specific binding proteins are therefore physiologically very important for neuronal functionality.

Diagnosis of Stroke and Neurodegenerative Diseases

Stroke or other neurodegenerative diseases develop and are a consequence of an overly active NMDA receptor at the neuronal synaptic junction. The active NMDA receptor stimulates influx of calcium ions into the cell and through

the effect of calmodulin it activates nNOS, which in turn increases production of NO in the neuron. Neuronal NO either causes or at least participates in development of neurotoxic injury, including stroke.

According to the findings of this invention, the increased activity of the NMDA receptor is to a certain extent dependent on binding of NMDA to nNOS through PDZ domains of nNOS binding proteins PSD-95 or PSD-93. This can be advantageously utilized for early detection of impending stroke or development of other neurodegenerative diseases by detecting a level of nNOS, PSD-95 or PSD-93 proteins.

When the level of nNOS is high, the probability of impending stroke or other neurodisturbance is high.

The detection of nNOS is according to Example 22 and as described above for diagnostic test for detection of muscular dystrophy. Instead of muscle tissue, brain or central nervous tissue biopsy is used. Alternatively, *in situ* imaging method is used using labeled PSD-95 protein inhibitors.

Treatment and Prophylaxis of Stroke and Other Neurodegenerative Diseases

Compounds that block association of nNOS with PSD-95 or PSD-93 are candidates for novel therapeutic agents useful for treatment or prophylaxis of stroke or other degenerative diseases. As shown, neuron-derived NO, associated with NMDA receptor activity, is responsible for and mediates brain injury following cerebral ischemia. Therefore, by blocking nNOS activity by disruption of its binding with binding proteins, the action of NO can be controlled and further damage to neurons is prevented.

Screening for drugs that block interaction of NMDA receptors with PSD-95 or PSD-93 could be done by an analogous procedure to that described in Example 23.

For this purpose, a 9-mer peptide identified as a SEQ.ID No. 3, corresponding to the final 9 amino acids of NMDA receptor 2B potently interacts with GST-fusion proteins encoding the first 2 PDZ domains of PSD-95 and PSD-93.

Therefore, a C-terminal 9-mer peptide of NMDA 2B

receptor, either radiolabeled or epitope tagged, can be advantageously used for a large scale screening assay for compounds which block its binding to PSD-95 or PSD-93. Those compounds would then be able to inhibit, or block by competition, the binding of PSD-95 or PSD-93 to nNOS.

One way how to produce these inhibitors is to label the peptide and to incubate it with PSD-95 or PSD-93 GST-fusion proteins to reach a binding equilibrium and immobilize the fusion proteins on glutathione resin. PSD-95 or PSD-93 fragments are retained by immobilized glutathione resin, and the resin is washed to elute unbound peptide. Using this assay one could perform large scale screening of compounds for drug discovery.

Potent inhibitors of this binding are therefore useful in treatment and in prevention of stroke and neurodegenerative disease. They would be administered in any suitable pharmaceutically acceptable route either before impending stroke or after the stroke developed, to prevent further neuronal damage.

Inhibitors of nNOS and Postsynaptic PSD-93 and PSD-95 Proteins

This invention also identifies a small 9-mer peptide that potently (Kd-1 μM) blocks association of nNOS with PSD-95 and PSD-93.

This invention demonstrates a novel mode to block brain damage due to nitric oxide, that is, the identification of small molecules that disrupt interaction of nNOS with PSD-95 and PSD-93. This invention demonstrates that such drugs would uncouple nNOS from neurotransmitter receptors and would prevent NO mediated brain damage.

Previous efforts to identify enzyme inhibitors of nNOS have been unsuccessful because there are two other isoforms of NOS (eNOS and iNOS), and nNOS selective drugs have not yet been identified. However, this invention clearly shows that only nNOS associates with PSD-95 and PSD-93, so that drugs which block these interactions would be specific for nNOS.

To that effect, a binding assay suitable for screening

for suitable inhibitors of nNOS/PSD-95 or PSD-93 protein has been developed and is described in Example 23.

Binding Assay for Screening Inhibitors of PSD-95 and PSD-93 Binding with nNOS

GST-fusion proteins linked to the first two or three PDZ motifs of PSD-95 or PSD-93 are expressed in E. coli as described in Example 6. Binding interactions to this fragment are monitored by a variety of assays known in the art. To detect binding of endogenous nNOS or NMDA receptor subunits to PSD-95 or PSD-93, pull down assays are done as described.

Screening for drugs that block interaction of NMDA receptors with PSD-95 or PSD-93 could be done by an analogous procedure as described above, and in Example 23.

UTILITY

Current invention is useful for diagnosis and treatment of muscular diseases, primarily for diagnosis and treatment of Duchenne dystrophy, Becker muscular dystrophy and other types of muscular dystrophies. Detection of presence or absence of nNOS in human biopsies, for example, immunohistochemically, detects the disease and its severity. Treatment of muscular dystrophies utilizes the restoration of fully functional dystrophin able to bind to nNOS, using, for example, gene therapy. Restoration of a functional dystrophin molecule to muscle represents a primary goal for therapy.

The invention is also useful for management of neurodegenerative diseases.

EXAMPLE 1

Tissue Extraction and Western Blot Analysis

This example describes methods used for skeletal muscle tissue extraction.

Mouse quadriceps skeletal muscle was homogenized in 10 vol (w/v) of buffer A (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM PMSF), and heavy microsomes were prepared by a standard protocol with minor modifications according to (J. Cell Biol., 96: 1008-16 (1983)). Nuclei were pelleted by centrifugation at 1000 x g. The supernatant was then centrifuged at 20,000 x g, yielding supernatant S_1. The

resulting heavy microsomal pellet was resuspended in buffer A containing 500 mM NaCl, incubated for 30 min at 4°C with agitation, and centrifuged at 15,000 x g, yielding supernatant S_2. The resulting pellet was resuspended in buffer A containing 500 mM NaCl plus 0.5% Triton X-100, incubated for 30 min at 4°C with agitation, and centrifuged at 15,000 x g, yielding supernatant S_3 and a final pellet, P.

Tissue extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide), and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). Membranes were incubated overnight with primary antisera bNOS, (1:250) and eNOS, (1:250), obtained from Transduction Laboratories; dystrophin (1:100), and spectrin (1:100), obtained from Novacastra Laboratories diluted in Tris-HCl-buffered saline containing 3% bovine serum albumin. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Amersham).

EXAMPLE 2

Affinity Chromatography

This example describes affinity chromatography used for sWGA affinity chromatography.

sWGA Sepharose Affinity Chromatography

Mouse quadriceps from wild-type and *mdx* mice were homogenized and solubilized in 10 vol of buffer B (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF) containing 1% digitonin. Solubilized membranes (4 mg) from wild-type and *mdx* mice were circulated for 1 hour with 250 μl of sWGA-agarose obtained from Vector Labs at 4°. Columns were washed sequentially with 5 ml of buffer B containing 0.1% digitonin, buffer B containing 0.1% digitonin and 500 mM NaCl, and buffer B containing 500 mM NaCl and 0.5% Triton X-100. Columns were affinity eluted with 1 ml of buffer B containing 0.3 M NAG with 0.1% digitonin. Chromatography of rat skeletal muscle followed a similar procedure, except 20 mg of solubilized membranes was loaded onto 1 ml of sWGA that was washed with 10 ml of buffers containing 500 mM NaCl and Triton X-100 and

eluted with 3 ml of 0.3 M N-methyl-D-glucosamine.

2',5'-ADP Affinity Chromatography

For 2',5'-ADP affinity chromatography, mouse quadriceps from wild-type and nNOS knockout mice were homogenized in 10 vol (w/v) of buffer B, and heavy microsomes were prepared and solubilized in buffer B containing 1% digitonin. Solubilized membranes (4 mg) from wild-type and nNOS knockout mice were applied to 150 μl columns of 2',5'-ADP-agarose (Sigma). Columns were sequentially washed with 5 ml of buffer B containing 0.1% digitonin, 1 ml of buffer B containing 500 mM NaCl and 0.1% digitonin, and 1 ml of buffer B containing 500 mM NaCl and 0.5% Triton X-100. Columns were affinity eluted with 1 ml of buffer B containing 20 mM NADPH and 0.1% digitonin.

EXAMPLE 3

Glutathione-S-Transferase-Fusion Proteins

This example describes preparation of glutathione-S-transferase (GST) fusion proteins.

A GST-nNOS(1-299) construct was generated by cloning sequences encoding the first 299 amino acids of rat brain NOS into the EcoRI site of the pGEX-2T vector. GST-fusion proteins were expressed in Escherichia coli and purified on glutathione-Sepharose beads according to Gene, 67:31-40 (1988) and according to the specifications of the manufacturer Pharmacia. Solubilized skeletal muscle membranes (2 mg) were incubated with control (GST) or GST-nNOS (1-299) beads for 1 hr. Beads were washed with buffer containing 0.5% Triton X-100 plus 300 mM NaCl, and proteins were eluted with 150 μl of loading buffer.

EXAMPLE 4

Dystrophin Immunoprecipitation

This example describes method used for dystrophin immunoprecipitation.

Monoclonal antibodies (2 μg) to dystrophin or Myc epitope (BABCO) were added to 1 ml aliquots of NAG eluate (15 μg), and samples were incubated on ice for 1 hour. Rabbit anti-mouse IgG (10μg) obtained from Cappel was then added, and after 30

min, 50 μl of protein A-Sepharose was used to precipitate antibodies. Protein A pellets were washed three times with buffer containing 200 mM NaCl and 0.1% Triton X-100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE.

EXAMPLE 5

Immunohistochemical and Immunoblotting Procedures

This example describes methods used for preparation of, skeletal muscle samples for immunohistochemical and immunoblotting procedures.

Unfixed skeletal muscle samples were flash frozen in liquid nitrogen cooled isopentane, sectioned on a cryostat (10 μm), and melted directly onto glass slides. Sections were then postfixed in 2% paraformaldehyde-phosphate-buffered saline (PBS). Tissues were blocked in PBS containing 1% normal goat serum. Monoclonal antibodies to dystrophin (1:200) obtained from Sigma, nNOS (1:100) obtained from Transduction Laboratories, spectrin (1:50) obtained from Novacastra Laboratories, or a polyclonal nNOS antibody (1:250) prepared according to Nature, 372: 546-548 (1994) were applied to sections overnight at 4° For indirect immunofluorescence, secondary goat anti-rabbit fluorescence isothiocyanate (FITC) or donkey anti-mouse Cy-3 conjugated antibodies were used according to the specifications of the manufacturer (1:200), Jackson Laboratories.

EXAMPLE 6

Mammalian Cell Transfections

This example illustrates the method used for mammalian cell transfection.

nNOS cDNAs were cloned into the mammalian expression vector pcDNA-3 obtained from Invitrogen. Monkey COS cells were grown in culture medium consisting of DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum. Cells were plated in 10 cm dishes at a density of 2 x 10^4 per square centimeter and transfected the following day using calcium phosphate as previously described in Nature, 351:714-718. Cells were washed with PBS 3 days following transfection, harvested in

2 ml of buffer containing 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and disrupted using a polytron.

EXAMPLE 7

Nitric Oxide Synthase Catalytic Assays

This example describes procedure used for assessment of for NOS catalytic activity.

Quadriceps skeletal muscle from wild-type and *mdx* mouse were homogenized in 10 vol of buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1mM EGTA, and 0.1 M NaCl. The homogenate was centrifuged at 20,000 x g, yielding the soluble fraction. The pellet was extracted in the same buffer containing 0.5 M NaCl and centrifuged at 20,000 x g, yielding the particulate fraction. Aliquots from these fractions were assayed in 125 µl reactions containing 100,000 cpm, of [^{3}H]arginine (60 Ci/mmol), 1 mM NADPH, 400 µM free calcium, 1 µM calmodulin, 3 µM each of tetrahydrobiopterin, FAD, and FMN. After incubation for 25 min at 22°C, assays were terminated with 4 ml of H_2O. Samples were applied to 0.5 ml Dowex AG50WX-8 (Na^+ form) columns. [^{3}H] citruline was quantified by liquid scintillation spectroscopy of the 4 ml flowthrough. Crude homogenates of transfected COS cells were assayed using an identical procedure.

EXAMPLE 8

Characterization of Human Tissues

This example describes methods used for evaluation of the nNOS localization in human tissue.

Human tissues were obtained from the pathology department at University of California, San Francisco. Clinical diagnosis of DMD was made on the basis of onset and progression of disease, the presence of creatinine kinase in serum, and histologic study of the biopsied muscles.

Tissues were snap frozen in liquid nitrogen-cooled isopentane. For Western blotting, cryostat sections were collected into plastic tubes and sonicated in buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF. Crude protein samples (200 µg per lane) were

resolved by SDS-PAGE and analyzed by Western blotting as described in Example 1. Immunohistochemistry was performed on 10 μm human tissue samples as described in Example 5. To ensure specificity, immuno-fluorescent nNOS staining was performed using two independently raised antisera. The first antiserum (α-nNOS1) reacts only with determinants in the N-terminal domain of nNOS (Neuron, 13:301-313, 1994b), while the second (α-nNOS2) reacts only with the C-terminal region, α-nNOS was obtained from Transduction Laboratories. Unless otherwise noted, all histologic sections were labeled with α-nNOS1.

EXAMPLE 9

Preparation of Polyclonal and Monoclonal Antibodies

This example lists a primary antibodies used for sarcolemmal studies.

In the studies directed to selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy, the following primary antibodies were used:

nNOS polyclonal antibody raised against homogenous nNOS protein purified from rat cerebellum, prepared according to Nature, 347: 768-770 (1990).

nNOS monoclonal antibody obtained from Transduction Lbas.

α1-syntrophin polyclonal antibody prepared according to Neuroreport, 5: 1577-1580 (1994).

Dystrophin monoclonal antibody was obtained from Sigma. β-dystroglycan monoclonal, utrophin monoclonal, and αsarcoglycan monoclonal antibodies were obtained from Novacastra.

EXAMPLE 10

Immunofluorescence Assay

This example describes the assay used for immunofluorescent studies on skeletal muscle samples from Becker muscular dystrophy patients and transgenic mice.

Unfixed skeletal muscle samples obtained from human patients or from mdx or transgenic mice were flash frozen in liquid nitrogen cooled isopentane, sectioned on a cryostat (10 μm) and melted directly onto glass slides. Sections were then

post-fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) or cold acetone. Tissues were "blocked" in PBS containing 1% normal goat serum. Primary antibodies were diluted in blocking reagent and were applied to sections overnight at 4°C. For indirect immunofluorescence secondary goat anti-rabbit FITC (1:200), or donkey anti-mouse Cy-3 (1:200) conjugated antibodies were used according to the manufacturer's specifications (Jackson Laboratories). Cy-3 conjugated α-BGT was diluted together with the secondary antibody for double labeling motor endplates.

EXAMPLE 11

Antibodies Immunoprecipitation

This example describes procedure used for precipitation of polyclonal antibodies to α1-syntrophin.

Polyclonal antibodies (1 μg) to α1-syntrophin or non-immune serum were added to 0.5 ml aliquots of solubilized skeletal muscle membranes (2 mg/ml) or muscle cytosol (1 mg/ml), and samples were incubated on ice for 1 hour. Protein A sepharose (50 μl) was used to precipitate antibodies. Protein A pellets were washed 3 times with buffer containing 100 mM NaCl and 1% tritox X-100. Immunoprecipitated proteins were denatured with a loading buffer and resolved by SDS-PAGE.

EXAMPLE 12

Fusion Protein Affinity Chromatography

This example describes fusion protein affinity chromatography procedure used for GST-nNOS (1-299) fusion protein.

A fusion protein of GST fused to the first 299 amino acids of nNOS was expressed in *Escherichia coli* and purified on glutathione sepharose beads as described Cell, 82: 743-752 (1995). Solubilized skeletal muscle membranes were incubated with control (GST) or GST-nNOS (1-299) beads. Samples were loaded into disposable columns, which were washed with 50 volumes of buffer containing 0.5% triton X-100 + 300 mM NaCl, and proteins eluted with 150 μl of SDS/PAGE loading buffer.

EXAMPLE 13

Antibodies and Western Blotting Used for Studies of Interaction of NOS with PSD-95 and α1-Syntropin

This example lists specific antibodies used for studies of interaction of NOS with the synaptic density protein PSD-95 and α1-syntropin.

The following primary antibodies were used:

nNOS1 polyclonal antibody raised against homogenous nNOS protein purified from rat cerebellum as described in Example 9.

nNOS monoclonal and eNOS monoclonal antibodies were obtained from Transduction Labs.

α1-syntrophin polyclonal as described in Example 9, PSD-95 polyclonal were prepared according to Neuron, 9: 929-942 (1992).

K$_v$1.4 polyclonal antibodies were prepared according to Nature, 378: 85-88 (1995).

c-myc monoclonal antibody 9E10 was obtained from BABCO.

T7-Tag monoclonal and GST 12 monoclonal antibodies were obtained for Santa Cruz Biotechnology, Inc.

For Western blotting, protein extracts were resolved by SDS/PAGE and transferred to PVDF membranes. Primary antibodies were diluted in block solution containing 3% BSA, 0.1% Tween-20 in TBS and incubated with membranes overnight at 4° Labeled bands were visualized using ECL. (Amersham). All nNOS Western blots used in nNOS monoclonal antibodies.

EXAMPLE 14

In situ hybridization

This example describes the procedure used for *in situ* hybridization used for coexpression of nNOS and PSD-95 transcripts.

In situ hybridization used ^{35}S-labeled RNA probes as described in Methods Enzymol., 225: 384-404 (1993). Antisense probes to nNOS (nucleotides 4119-5057, PSD-95 (1-1155), PSD 93 (237-927) or sense control PSD-93 (237-927) were synthesized from bluescript vectors.

EXAMPLE 15

PSD-95 and nNOS Colocalization-Immunochemistry Assay

This example describes immunohistochemistry assay used in studies of the association of nNOS with PSD-95 in neuronal populations and during embryonic development.

Rats were perfused with 4% paraformaldehyde, tissues were harvested, post-fixed at 4°C for 3 hours, and cryoprotected in 20% sucrose overnight. Twenty micron sections were cut on a cryostat and melted onto glass slides (Plus), obtained from Fisher. Sections were then blocked for one hour in a buffer containing 2% goat serum, 0.1% triton X-100 in PBS. Primary antibodies to nNOS (polyclonal nNOS), PSD 95, or α1-syntrophin were diluted into a blocking reagent and incubated with sections overnight. Immunoperoxidase histochemistry was performed using the ABC method according to a kit obtained from Vector. Immunofluorescent staining of rat extensor digitorum longus muscle was done as described in Cell, 82: 743-752 (1995). Control sections lacking primary antisera were stained in parallel.

EXAMPLE 16

Cell Culture and Transfection

This example describes assays used for cell culture and cloning of nNOS and transfection assays.

Neuronal NOS cDNA were cloned into the EcoRV and Xba I sites of the mammalian expression vector pcDNA 3 (Invitrogen). 5'a and 5'b containing constructs were amplified by PCR, sequenced, and cloned into the unique Nar I restriction site of nNOS. PSD-95-myc construct containing amino acids 1-386 with a C-terminal myc-epitope tag was amplified by PCR and cloned into the BamHI and EcoRI sites of pcDNAIII. Monkey COS cells were grown and transfected using calcium phosphate as previously described in Nature, 351: 714-718 (1991). Two days following transfection cells were washed with PBS, harvested in 2 ml of buffer containing 25 mM TrisHCl pH 7.4, 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF and disrupted using a polytron.

EXAMPLE 17

Immunoprecipitations of PSD-95 Proteins

This example describes procedure used for immunoprecipitation of PSD-95 proteins.

Rat cerebellum was homogenized in 20 volumes of a buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl and centrifuged at 100,000x g to yield cytosol. Cerebellar membranes were solubilized with 1% digitonin and 100 mM NaCl and centrifuged to remove the insoluble cytoskeleton. Three μl of PSD-95 polyclonal antiserum to PSD-95 or 3 μl non-immune serum were added to 1 ml (500 μg) of cerebellar cytosol or solubilized membranes. After a 60 minute incubation on ice, 50 μl of protein A sepharose was added to precipitate antibodies. Protein A pellets were washed 3 times with a buffer containing 200 mM NaCl and 1% triton X-100. Immunoprecipitated proteins were denatured with a loading buffer and resolved by SDS-PAGE. Heavy microsomes of rat gastrocnemius were prepared and solubilized with 1% triton X-100 as described in Cell, 82: 743-752 (1995). Five μg polyclonal antiserum to α1-syntrophin or non-immune serum were added to 1 ml (500 μg) solubilized muscle samples. Immunoprecipitations from transfected COS cells used polyclonal antibody to nNOS.

EXAMPLE 18

GST Fusion Protein Affinity Chromatography

This example describes methods used for construction of GST fusion constructs.

GST fusion constructs were constructed by PCR and fusion proteins purified as described in Example 3. For "pull down" assays, solubilized tissue samples were incubated with control or GST-fusion protein beads for 1 hour. Beads were washed with a buffer containing 0.5% triton X-100 and 350 mM NaCl, and proteins were eluted with SDS loading buffer. NMDA receptor peptide (SEQ ID NO: 7:) (lys leu ser ser ile glu ser asp val) or control peptide (SEQ ID NO: 8:) (lys pro lys his ala lys his pro asp gly his ser gly asn leu cys) were added where indicated during tissue incubation with the fusion protein.

EXAMPLE 19

Generation of α1-Syntrophin Fusion Proteins and Protein Overlay Assay

This example describes production of α1-syntrophic fusion proteins and procedures used therefore.

cDNAs encoding mouse α1-syntrophin domains (PH1a domain, amino acids 1-77; PH1b, 162-271; PDZ, 75-170; PH2, 281-402; SU domain 401-499) were amplified by PCR and cloned into pET28a vector (Novagen, Inc.) with the exception of PH1a and PH1b that were ligated together to produce the intact PH1 domain.

Clones were sequenced and electroporated into BL21 (λDE3)pLysS cells. Overnight cultures were diluted 1:10, incubated 2 hours and induced for 3 hours with IPTG. Expressed proteins, which contain a T7.Tag epitope encoded in the vector, were purified on Nickel columns obtained from Novagen, Inc. The PH1 and PDZ domains were purified from the soluble friction; PH2 and SU were purified from urea solubilized inclusions. Fusion proteins were separated on 15% SDS/PAGE gels, transferred to nitrocellulose membranes, blocked with 5% skim milk in 25 mM Tris (pH 7.5), 150 mM NaCl and 0.1% tween-20 (TBS-Tween) and incubated with purified fusion proteins (20 μg/ml) in this buffer for 1 hour at 25°C. Blots were washed 3 x 10 minutes in TBS-Tween, incubated with primary antibody T7.Tag or GST for 30 minutes and bands visualized by ECL.

EXAMPLE 20

mRNA Isolation and cDNA Analysis

This example describes procedures used for isolation of mRNA and cDNA analysis.

RNA was isolated using the guanidine isothiocyanate/CsCl method and mRNA was selected using oligo dT sepharose. For Northern blotting, mRNA was separated on a formaldehyde agarose gel and transferred to a Nylon membrane. A random primed probe ^{32}P probe was generated using the full-length (5057 bp) nNOS cDNA as described in Nature, 351: 714-718 (1991) as a template. The filter was washed at high

stringency, 68°C, 0.1% SSC, 0.1% SDS and exposed to X-ray film overnight at -70°C.

Thermal RACE-PCR was performed as described in Method Enzymol., 218: 340-356 (1993). The sequence of the nNOS specific primers in exon 3 were:

Race 1: SEQ ID NO: 9:

Race 2: SEQ ID NO: 10:

For RT-PCR, mRNA was reverse transcribed with RTth polymerase using random hexamer primers. The sequence of the PCR primers used were:

P1: SEQ ID NO: 11:

P2: SEQ ID NO: 12:

P3: SEQ ID NO: 13:

P4: SEQ ID NO: 14:

Clones encoding PSD-93 were isolated from a rat brain cDNA library (Stratagene) by plaque hybridization.

EXAMPLE 21

nNOS Protein Purification and Catalytic Assays

This example describes purification procedure used for solubilized tissue homogenated and nNOS protein catalytic assays.

Solubilized tissue homogenates were incubated with 100 μl of 2'5'-ADP agarose (Sigma), columns were washed with 5 ml of buffer containing 0.35 M NaCl, and were eluted with 10 mM NADPH. Catalytic NOS activity was quantitated by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline as described in PNAS USA, 87:682-685 (1990).

EXAMPLE 22

Histologic Analysis of Nitric Oxide Synthase as a Diagnostic Test for Muscle Disease

This example describes a diagnostic test useful for detecting muscle disease.

Human muscle biopsies are harvested and span frozen, and cryosectioned according to a standard protocol. Sarcolemmal localization of nNOS is detected by either immunofluorescence analysis or by histochemical staining for NADPH diaphorase, which reflects nNOS activity according to Nature; 347:768-770

(1991).

nNOS immunofluorescence is performed as described in Example 10. Briefly, nNOS antibodies are applied to cryostat sections of muscle samples overnight at 4°C. Secondary goat anti-rabbit Cy-3 conjugated antibodies (1:200) are obtained from Jackson Laboratories and are used according to the manufacturer's specifications.

NADPH diaphorase staining is performed as described in Nature, ibid. Briefly, the cryosections are incubated with 1mM NADPH, 0.2 mM nitroblue tetrazolium in a 0.1 M Tris-HCl buffer (pH 7.4) containing 0.2% triton X-100 for 90 minutes at room temperature.

Presence of nNOS is detected by the presence of blue staining.

The presence of sarcolemmal nNOS staining is consistent with presence of a functional dystrophin molecule. The absence of sarcolemmal nNOS is a sensitive and specific indicator of abnormal dystrophins.

EXAMPLE 23

Binding Assay to Screen for Compounds that Disrupt Interaction of nNOS, NMDA Receptors or Other Ion Channels with PSD-95 or PSD-93

This example describes a binding assay useful for screening compounds which prevent, inhibit or disrupt binding of nNOS, NMDA receptors or other ion channels with PSD-95 and PSD-93 proteins.

GST fusion proteins linked to the first two or three PDZ motifs of PSD-95 or PSD-93 are expressed in E. coli as described in Example 6. Binding interactions to this fragment are monitored by a variety of assays known and used in teh art. To detect binding of endogenous nNOS or NMDA receptor subunits to PSD-05 or PSD-93 pull down assays are done as described below.

Larger scale screening assays are facilitated by expressing an appropriate N-terminal fragment of nNOS in a bacterial expression system. This fragment is radiolabeled or epitope tagged and binding of these fragment to expressed

PSD-95 or PSD-93 GST-fusion protein is monitored by a filtration binding assay.

nNOS 1-299 is expressed in E-coli with a N-terminal hexahistidine tag and a heart muscle protein kinase site. This fragment is radiolabeled with ^{32}P using [^{32}P] ATP and heart muscle kinase. PSD-95 or PSD-93 GST-fusion proteins are then incubated with the labeled nNOS fragment. After incubation to reach binding equilibrium, PSD-95 or PSD-93 fragments are retained by immobilized glutathione resin, and the resin is washed to elute unbound nNOS fragments. Bound nNOS fragments on the resin are quantitated by scintillation counting or by an ELISA. Using this assay, large scale screening of compounds for drug discovery is possible.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BREDT, DAVID S.
BRENMAN, JAY E.
CHAO, DANIEL S.

(ii) TITLE OF INVENTION: NITRIC OXIDE SYNTHASE BINDING PROTEINS AND A METHOD FOR THEIR USE IN TREATMENT AND DIAGNOSIS OF MUSCULAR DYSTROPHY

(iii) NUMBER OF SEQUENCES: 41

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette - 3.5 inch, 1.44 Kb storage
(B) COMPUTER: PC
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: Wordperfect 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/613,114
(B) FILING DATE: MARCH 8, 1996
(C) CLASSIFICATION: 424

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2963 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
CTAATTGAAA TTACTGGGCA TAATGCTATA TATAGCCAAT GAAGAGATTC TGAGCTCTCA   60
CTCAGTGCCT TCAAGACATG TCGTTTTGTA GTCAGAGGAA ACAGGGATCA ATGCATTTTC  120
AAACTGACAG AGGGAACGGA TGCTCCTTAG CAGCACATGC CCAGGATCGT GTGTGTGGGG  180
CTTGCGCTGT GCTGAGAAGC TGATCACCGG CCCATATGCC CCTTATTTAC TGCAATGTTC  240
TTTGCATGTT ATTGTGCACT CCGGACTAAC GTGAAGAAGT ATCGATACCA AGATGAGGAC  300
GGTCCACATG ATCATTCCTT ACCTCGGCTA ACTCATGAAG TAAGAGGTCC AGAACTGGTG  360
CATGTGTCGG AAAAGAACCT CTCTCAAATA GAAAATGTCC ACGGATATGT CTTACAGTCT  420
CACATTTCTC CTCTGAAGGC TAGCCCTGCT CCTATAATTG TCAACACAGA CACTTTGGAC  480
ACTATTCCTT ATGTCAATGG AACAGAAATT GAATATGAAT TTGAAGAAAT TACATTGGAG  540
AGGGGAAATT CAGGTCTGGG ATTCAGTATT GCTGGAGGGA CAGATAATCC TCACATTGGA  600
```

```
GATGACCCTG GCATATTTAT TACGAAGATT ATTCCAGGAG GTGCTGCAGC AGAGGATGGC  660
AGACTCAGGG TCAACGATTG TATCTTGCGG GTGAATGAAG TTGATGTGTC GGAGGTTTCC  720
CACAGTAAAG CAGTGGAGGC CCTCAAGGAA GCAGGCTCTA TTGTTCGACT GTATATACTT  780
AGAAGACGAC CCATCCTGGA GACTGTTGTG GAAATCAAAC TTTTCAAAGG GCCAAAAGGT  840
TTAGGCTTCA GTATTGCTGG AGGGGTGGGG AACCAGCACA TACCCGGAGA CAACAGCATT  900
TATGTAACGA AAATTATGGA TGGTGGAGCT GCACAGAAAG ATGGGAGGTT GCAAGTAGGA  960
GACAGACTGC TAATGGTAAA TAACTATAGT TTAGAAGAAG TTACACATGA AGAGGCTGTA 1020
GCGATATTGA AAAATACATC TGATGTTGTT TATCTAAAAG TTGGCAAACC CACAACCATT 1080
TATATGACTG ATCCTTATGG GCCACCGGAT ATCACTCACT CTTATTCTCC ACCAATGGAA 1140
AATCATCTAC TGTCTGGTAA CAATGGCACG TTAGAATACA AAACATCCCT GCCGCCCATC 1200
TCTCCAGGGA AGTACTCACC AATTCCAAAG CACATGCTGG TTGAAGATGA CTACACAAGG 1260
CCTCCGGAAC CTGTTTACAG CACTGTGAAT AAACTGTGTG ATAAACCTGC TTCTCCCAGG 1320
CACTATTCCC CTGTTGAGTG TGACAAAAGC TTCCTTCTCT CAACTCCTTA CCCCCACTAC 1380
CACCTAGGCC TGCTCCCTGA CTCTGACATG ACCAGTCATT CTCAGCACAG TACTGCAACT 1440
CGTCAGCCCT CAGTGACTCT CCAACGGGCC ATCTCCCTGG AAGGGGAGCC CCGAAAGGTG 1500
GTCCTTCACA AAGGCTCCAC TGGCCTGGGC TTCAACATTG TGGGTGGAGA AGACGGAGAA 1560
GGTATTTTTG TATCCTTCAT TCTGGCCGGT GGACCAGCAG ACCTGAGTGG GGAGCTCCAG 1620
AGAAGAAAAC AGATTTTATC GGTGAATGGT ATCCATCTCC CAGGAGACTC TCATGAACAG 1680
GCACTTCCCC TGAAGGGGGC GGGGCAGACA GTGACAATCA TAGCACAATA TCAACCTGAA 1740
GATTACTCTC GATTCGAGGC CAAAATCCAT GACCTACGAG AGCAGATGAT GAACCACAGC 1800
ATGAGTTCCG GGTCCGGGTC CCTTCGAACC AATCAGAAAC GCTCCCTGTA TGTCAGAGCC 1860
ATGTTTGACT ATGACAAGAG CAAGGACAGT GGACTGCCTA GCCAAGGACT TAGTTTTAAA 1920
TATGGAGACA TCCTTAATGT CATCAATGCC TCTGATGATG AGTGGTGGCA AGCCAGAAGG 1980
GTCATACAAG ATGGGGACAG CGAGGAGATG GGAGTCATTC CCAGCAAACG GAGGGTGGAA 2040
AGAAAGGAGC GTGCCCGATT GAAGACAGTG AAGTTCAATG CAAAACCTGG TGTGATTGAT 2100
TCCAAAGGGT CATTCAATGA CAAGCGTAAA AAGAGCTCCA TCTTTTCACG AAAATTCCCA 2160
TTCTACAAGA ACAAGGAGCA GAGTGAGCAG GAAACCAGTG ATCCTGAACG AGGACAAGAA 2220
GATCTCATTC TTTCCTATGA ACCTGTCACG AGGCAGGAAA TAAACTACAC CCGACCAGTG 2280
ATTATCCTGG GCCCCATGAA GGATCGAATC AATGATGACT TGATATCTGA ATTTCCTGAT 2340
AAATTTGGCT CCTGTGTGCC TCATACTACG AGGCCAAAGC GTGACTACGA AGTCGACGGC 2400
AGAGACTATC ACTTTGTCAT TTCTAGAGAA CAAATGGAGA AAGATATCCA AGAGCACAAA 2460
TTTATAGAAG CCGGCCAGTA CTATGACAAT TTATATGGAA CCAGTGTGCA GTCTGTGAGA 2520
TTTGTAGCAG AAAGGGGCAA ACACTGTATA CATGATGTAT CGGGAAATGC TATTAAGCGG 2580
TTACAAGTTG CCCAGCTCTA TCCCATTGCT ATCTTCATAA AGCCCAAGTC TCTGGAACCT 2640
CTGATGGAGA TGAATAACGG TCTAATGGAG GAACAAGCCA AGAAAACCTA TGACCGGGCA 2700
ATTAAGCTAG AACAAGAATT TGGAGAATAT TTTACAGCTA TTGTCCAAGG AGATACCTTA 2760
GAAGATATTT ACAACCAATG CAAGCTTGTT ATTGAAGAGC AGTCTGGACC TTTCATCTGG 2820
ATTCCCTCAA AGGAGAAGTT ATAAATTAGC TACTGCACCT CTGACAACGA CGAAGAGCAT 2880
ATAGAAGAAC AAATATATAT AATATACACT GAGGCTTTAT GTTTTGTTGC ATTATGTTTT 2940
GCAGTCAATG TGAATCTTAT GAA                                          2963
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

leu ile glu ile thr gly his asn ala ile tyr ser gln
1 5 10 13

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

arg asp ser glu leu ser leu ser ala phe lys thr cys arg phe

1 5 10 15
val val arg gly asn arg asp gln cys ile phe lys leu thr glu
20 25 30
gly thr asp ala pro
35

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

gln his met pro arg ile val cys val gly leu ala leu cys
1 5 10 14

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 882 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

glu ala asp his arg pro ile cys pro leu phe thr ala met phe
1 5 10 15
phe ala cys tyr cys ala leu arg thr asn val lys lys tyr arg
20 25 30
tyr gln asp glu asp gly pro his asp his ser leu pro arg leu
35 40 45
thr his glu val arg gly pro glu leu val his val ser glu lys
50 55 60
asn leu ser gln ile glu asn val his gly tyr val leu gln ser
65 70 75
his ile ser pro leu lys ala ser pro ala pro ile ile val asn
80 85 90
thr asp thr leu asp thr ile pro tyr val asn gly thr glu ile
95 100 105
glu tyr glu phe glu glu ile thr leu glu arg gly asn ser gly
110 115 120
leu gly phe ser ile ala gly gly thr asp asn pro his ile gly
125 130 135
asp asp pro gly ile phe ile thr lys ile ile pro gly gly ala
140 145 150
ala ala glu asp gly arg leu arg val as asp cys ile leu arg
155 165
val asn glu val asp val ser glu val s his ser lys ala val
170 1 180
glu ala leu lys glu ala gly ser ile va arg leu tyr ile leu
185 190 195
arg arg arg pro ile leu glu thr val val glu ile lys leu phe
200 205 210
lys gly pro lys gly leu gly phe ser ile ala gly gly val gly
215 220 225
asn gln his ile pro gly asp asn ser ile tyr val thr lys ile
230 235 240
met asp gly gly ala ala gln lys asp gly arg leu gln val gly
245 250 255
asp arg leu leu met val asn asn tyr ser leu glu glu val thr

260 265 270
his glu glu ala val ala ile leu lys asn thr ser asp val val
275 280 285
tyr leu lys val gly lys pro thr thr ile tyr met thr asp pro
290 295 300
tyr gly pro pro asp ile thr his ser tyr ser pro pro met glu
305 310 315
asn his leu leu ser gly asn asn gly thr leu glu tyr lys thr
320 325 330
ser leu pro pro ile ser pro gly lys tyr ser pro ile pro lys
335 340 345
his met leu val glu asp asp tyr thr arg pro pro glu pro val
350 355 360
tyr ser thr val asn lys leu cys asp lys pro ala ser pro arg
365 370 375
his tyr ser pro val glu cys asp lys ser phe leu leu ser thr
380 385 390
pro tyr pro his tyr his leu gly leu leu pro asp ser asp met
395 400 405
thr ser his ser gln his ser thr ala thr arg gln pro ser val
410 415 420
thr leu gln arg ala ile ser leu glu gly glu pro arg lys val
425 430 435
val leu his lys gly ser thr gly leu gly phe asn ile val gly
440 445 450
gly glu asp gly glu gly ile phe val ser phe ile leu ala gly
455 460 465
gly pro ala asp leu ser gly glu leu gln arg arg lys gln ile
470 475 480
leu ser val asn gly ile his leu pro gly asp ser his glu gln
485 490 495
ala leu pro leu lys gly ala gly gln thr val thr ile ile ala
500 505 510
gln tyr gln pro glu asp tyr ser arg phe glu ala lys ile his
515 520 525
asp leu arg glu gln met met asn his ser met ser ser gly ser
530 535 540
gly ser leu arg thr asn gln lys arg ser leu tyr val arg ala
545 550 555
met phe asp tyr asp lys ser lys asp ser gly leu pro ser gln
560 565 570
gly leu ser phe lys tyr gly asp ile leu asn val ile asn ala
575 580 585
ser asp asp glu trp trp gln ala arg arg val ile gln asp gly
590 595 600
asp ser glu glu met gly val ile pro ser lys arg arg val glu
605 610 615
arg lys glu arg ala arg leu lys thr val lys phe asn ala lys
620 625 630
pro gly val ile asp ser lys gly ser phe asn asp lys arg lys
635 640 645
lys ser phe ile phe ser arg lys phe pro phe tyr lys asn lys
650 655 660
glu gln ser glu gln glu thr ser asp pro glu arg gly gln glu
665 670 675
asp leu ile leu ser tyr glu pro val thr arg gln glu ile asn
680 685 690
tyr thr arg pro val ile ile leu gly pro met lys asp arg ile
695 700 705
asn asp asp leu ile ser glu phe pro asp lys phe gly ser cys
710 715 720
val pro his thr thr arg pro lys arg asp tyr glu val asp gly
725 730 735
arg asp tyr his phe val ile ser arg glu gln met glu lys asp
740 745 750
ile gln glu his lys phe ile glu ala gly gln tyr tyr asp asn
755 760 765

```
leu tyr gly thr ser val gln ser val arg phe val ala glu arg
                770                 775                 780
gly lys his cys ile his asp val ser gly asn ala ile lys arg
                785                 790                 795
leu gln val ala gln leu tyr pro ile ala ile phe ile lys pro
                800                 805                 810
lys ser leu glu pro leu met glu met asn asn gly leu met glu
                815                 820                 825
glu gln ala lys lys thr tyr asp arg ala ile lys leu glu gln
                830                 835                 840
glu phe gly glu tyr phe thr ala ile val gln gly asp thr leu
                845                 850                 855
glu asp ile tyr asn gln cys lys leu val ile glu glu gln ser
                860                 865                 870
gly pro phe ile trp ile pro ser lys glu lys leu
                875                 880     882
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
ile ser tyr cys thr ser asp asn asp glu glu his ile glu glu
 1               5                  10                  15
gln ile tyr ile ile tyr thr glu ala leu cys phe val ala leu
                20                  25                  30
cys phe ala val asn val asn leu met
                35                  39
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
lys leu ser ser ile glu ser asp val
 1               5                   9
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acid
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
lys pro lys his ala lys his pro asp gly his ser gly asn leu cys
 1               5                  10                  15  16
```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCACAGATCA TTGAAGACTC G 21

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGGGAATTCC CCGCCCCAGG GGCGGGGAGC TTT 33

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTCCCTGCGT ATTGATGCA 19

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCCGACCTG AGATTCCC 18

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTCTGCATCT GTCAAGCTGG 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCTTCACCAG GAAGCCCAGA 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

met phe phe ala cys tyr cys ala leu arg thr asn val lys lys
1 5 10 15
tyr arg tyr gln asp glu asp gly pro his
20 25

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

asp his ser leu pro arg leu thr his glu val arg gly pro glu
1 5 10 15
leu val his val ser glu lys asn leu ser gln ile glu asn val
20 25 30
his gly tyr val leu gln ser his ile ser pro leu lys ala ser
35 40 45
pro ala pro ile ile val asn thr asp thr leu asp thr ile pro
50 55 60
tyr
61

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid

(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
val asn gly thr glu ile glu tyr glu phe glu glu ile thr leu
 1               5                  10                  15
glu arg gly asn ser gly leu gly phe ser ile ala gly gly thr
            20                  25                  30
asp asn pro his ile gly asp asp pro gly ile phe ile thr lys
        35                  40                  45
ile ile pro gly gly ala ala ala glu asp gly arg leu arg val
    50                  55                  60
asn asp cys ile leu arg val asn glu val asp val ser glu val
65                  70                  75
ser his ser lys ala val glu ala leu lys glu ala gly ser ile
            80                  85                  90
val arg leu tyr ile leu arg arg arg pro ile leu glu thr val
        95                  100                 105
val glu ile lys leu phe lys gly pro lys gly leu gly phe ser
    110                 115                 120
ile ala gly gly val gly asn gln his ile pro gly asp asn ser
125                 130                 135
ile tyr val thr lys ile met asp gly gly ala ala gln lys asp
            140                 145                 150
gly arg leu gln val gly asp arg leu leu met val asn asn tyr
        155                 160                 165
ser leu glu glu val thr his glu glu ala val ala ile leu lys
    170                 175                 180
asn thr ser asp val val tyr leu lys val gly lys pro thr thr
185                 190                 195
ile tyr met thr asp pro tyr gly pro pro asp ile thr his ser
            200                 205                 210
tyr ser pro pro met glu asn his leu leu ser gly asn asn gly
        215                 220                 225
thr leu glu tyr lys thr ser leu pro pro ile ser pro gly lys
    230                 235                 240
tyr ser pro ile pro lys his met leu val glu asp asp tyr thr
245                 250                 255
arg pro pro glu pro val tyr ser thr val asn lys leu cys asp
            260                 265                 270
lys pro ala ser pro arg his tyr ser pro val glu cys asp lys
        275                 280                 285
ser phe leu leu ser thr pro tyr pro his tyr his leu gly leu
    290                 295                 300
leu pro asp ser asp met thr ser his ser gln his ser thr ala
305                 310                 315
thr arg gln pro ser val thr leu gln arg ala ile ser leu glu
            320                 325                 330
gly glu pro arg lys val val leu his lys gly ser thr gly leu
        335                 340                 345
gly phe asn ile val gly gly glu asp gly glu gly ile phe val
    350                 355                 360
ser phe ile leu ala gly gly pro ala asp leu ser gly glu leu
365                 370                 375
gln arg arg lys gln ile leu ser val asn gly ile his leu pro
            380                 385                 390
gly asp ser his glu gln
        395 396
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 268 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
ala leu pro leu lys gly ala gly gln thr val thr ile ile ala
 1               5                  10                  15
gln tyr gln pro glu asp tyr ser arg phe glu ala lys ile his
            20                  25                  30
asp leu arg glu gln met met asn his ser met ser ser gly ser
        35                  40                  45
gly ser leu arg thr asn gln lys arg ser leu tyr val arg ala
    50                  55                  60
met phe asp tyr asp lys ser lys asp ser gly leu pro ser gln
65                  70                  75
gly leu ser phe lys tyr gly asp ile leu asn val ile asn ala
                80                  85                  90
ser asp asp glu trp trp gln ala arg arg val ile gln asp gly
            95                  100                 105
asp ser glu glu met gly val ile pro ser lys arg arg val glu
        110                 115                 120
arg lys glu arg ala arg leu lys thr val lys phe asn ala lys
    125                 130                 135
pro gly val ile asp ser lys arg gly gln glu asp leu ile leu
140                 145                 150
ser tyr glu pro val thr arg gln glu ile asn tyr thr arg pro
155                 160                 165
val ile ile leu gly pro met lys asp arg ile asn asp asp leu
            170                 175                 180
ile ser glu phe pro asp lys phe gly ser cys val pro his thr
        185                 190                 195
thr arg pro lys arg asp tyr glu val asp gly arg asp tyr his
    200                 205                 210
phe val ile ser arg glu gln met glu lys asp ile gln glu his
215                 220                 225
lys phe ile glu ala gly gln tyr tyr asp asn leu tyr gly thr
            230                 235                 240
ser val gln ser val arg phe val ala glu arg gly lys his cys
        245                 250                 255
ile his asp val ser gly asn ala ile lys arg leu gln
    260                 265         268
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
val ala gln leu tyr pro ile ala ile phe ile lys pro lys ser
 1               5                  10                  15
leu glu pro leu met glu met asn asn gly leu met glu g u gln
            20                  25                  30
ala lys lys thr tyr asp arg ala ile lys leu glu gln giu phe
        35                  40                  45
gly glu tyr phe thr ala ile val gln gly asp thr leu glu asp
    50                  55                  60
ile tyr asn gln cys lys leu val ile glu glu gln ser gly pro
65                  70                  75
phe ile trp ile pro ser lys glu lys leu
                80                  85
```

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 20:

```
met asp cys leu cys ile val thr thr
 1               5                   9
```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

```
lys lys tyr arg tyr gln asp glu asp thr pro pro leu glu his
 1               5                  10                  15
ser pro ala his leu
            20
```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 280 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22

```
pro asn gln ala asn ser pro pro val ile val asn thr asp thr
 1               5                  10                  15
leu glu ala pro gly tyr glu leu gln val asn gly thr glu gly
            20                  25                  30
glu met glu tyr glu glu ile thr leu glu arg gly asn ser gly
        35                  40                  45
leu gly phe ser ile ala gly gly thr asp asn pro his ile gly
    50                  55                  60
asp asp pro ser ile phe ile thr lys ile ile pro gly gly ala
65                  70                  75
ala ala gln asp gly arg leu arg val asn asp ser ile leu phe
            80                  85                  90
val asn glu val asp val arg glu val thr his ser ala ala val
        95                 100                 105
glu ala leu lys glu ala gly ser ile val arg leu tyr val met
    110                 115                 120
arg arg lys pro pro ala glu lys val met glu ile lys leu ile
125                 130                 135
lys gly pro lys gly leu gly phe ser ile ala gly gly val gly
            140                 145                 150
asn gln his ile pro gly asp asn ser ile tyr val thr lys ile
        155                 160                 165
ile glu gly gly ala ala his lys asp gly arg leu gln ile gly
    170                 175                 180
asp lys ile leu ala val asn ser val gly leu glu asp val met
185                 190                 195
his glu asp ala val ala ala leu lys asn thr tyr asp val val
```

```
                200                 205                 210
tyr leu lys val ala lys pro ser asn ala tyr leu ser asp ser
                215                 220                 225
tyr ala pro pro asp ile thr thr ser tyr ser gln his leu asp
                230                 235                 240
asn glu ile ser his ser ser tyr leu gly thr asp tyr pro thr
                245                 250                 255
ala met thr pro thr ser pro arg arg tyr ser pro val ala lys
                260                 265                 270
asp leu leu gly glu glu asp ile pro arg
                275                 280
```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 203 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```
glu pro arg arg ile val ile his arg gly ser thr gly leu gly
 1               5                  10                  15
phe asn ile val gly gly glu asp gly glu gly ile phe ile ser
            20                  25                  30
phe ile leu ala gly gly pro ala asp leu ser gly glu leu arg
            35                  40                  45
lys gly asp gln ile leu ser val asn gly val asp leu arg asn
            50                  55                  60
ala ser his glu gln ala ala ile ala leu lys asn ala gly gln
            65                  70                  75
thr val thr ile ile ala gln tyr lys pro glu glu tyr ser arg
            80                  85                  90
phe glu ala lys ile his asp leu arg glu gln leu met asn ser
            95                  100                 105
ser leu gly ser gly thr ala ser leu arg ser asn pro lys arg
            110                 115                 120
gly phe tyr ile arg ala leu phe asp tyr asp lys thr lys asp
            125                 130                 135
cys gly phe leu ser gln ala leu ser phe arg phe gly asp val
            140                 145                 150
leu his val ile asp ala gly asp glu glu trp trp gln ala arg
            155                 160                 165
arg val his ser asp ser glu thr asp asp ile gly phe ile pro
            170                 175                 180
ser lys arg arg val glu arg arg glu trp ser arg leu lys ala
            185                 190                 195
lys asp trp gly ser ser ser gly
            200             203
```

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```
ser gln gly arg glu asp ser val leu ser tyr glu thr val thr
 1               5                  10                  15
gln met glu val his tyr ala arg pro ile ile ile leu gly pro
            20                  25                  30
thr lys asp arg ala asn asp asp leu leu ser glu phe pro asp
```

```
                35                  40                  45
lys phe gly ser cys val pro his thr thr arg pro lys arg glu
                50                  55                  60
tyr glu ile asp gly arg asp tyr his phe val ser ser arg glu
        65                  70                  75
lys met glu lys asp ile gln ala his lys phe ile glu ala gly
                80                  85                  90
gln tyr asn ser his leu tyr gly thr ser val gln ser val arg
                95                  100                 105
glu val ala glu gln gly lys his cys ile leu asp val ser ala
                110                 115                 120
asn ala val arg arg leu gln
                125     127
```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
ala ala his leu his pro ile ala ile phe ile arg pro arg ser
 1                  5                  10                  15
leu glu asn val leu glu ile asn lys arg ile thr glu glu gln
                20                  25                  30
ala arg lys ala phe asp arg ala thr lys leu glu gln glu phe
                35                  40                  45
thr glu cys phe ser ala ile val glu gly asp ser phe glu glu
                50                  55                  60
ile tyr his lys val lys arg val ile glu asp leu ser gly pro
                65                  70                  75
tyr ile trp val pro ala arg glu arg leu
                80                  85
```

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
val arg leu phe lys arg lys val gly gly leu gly phe leu val
 1                  5                  10                  15
lys
 16
```

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
glu arg val ser lys pro pro val ile ile ser asp leu ile arg
  1               5                  10                  15
gly gly ala ala glu gln ser gly leu ile gln ala gly asp ile
            20                  25                  30
ile leu ala val asn asp arg pro leu val asp leu ser tyr asp
        35                  40                  45
ser ala leu glu val leu
    50  51
```

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

```
val arg ile val lys gln glu ala gly gly leu gly ile ser ile
  1               5                  10                  15
lys gly gly
      18
```

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
arg glu asn his met pro ile leu ile ser lys ile phe arg gly leu
 1               5                  10                  15
ala ala glu gln ser arg leu leu phe val gly asp ala ile leu ser
            20                  25                  30
val asn gly thr asp leu arg asp ala thr his asp gln ala val gln
        35                  40                  45
ala leu
     47
```

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
val arg val val lys gln glu ala gly gly leu gly ile ser ile
 1               5                  10                  15
lys gly gly
      18
```

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```
arg glu asn arg met pro ile leu ile ser lys ile phe pro gly
 1               5                  10                  15
leu ala ala asp gln ser arg ala leu arg leu gly asp ala ile
            20                  25                  30
leu ser val asn gly thr asp leu arg gln ala thr his asp gln
        35                  40                  45
ala val gln ala leu
    50
```

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
arg ile val ile his arg gly ser thr gly leu gly phe asn ile
 1               5                  10                  15
val gly gly
      18
```

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
glu asp gly glu gly ile phe ile ser phe ile leu ala gly gly
 1               5                  10                  15
pro ala asp leu ser gly glu leu arg lys gly asp gln ile leu
            20                  25                  30
ser val asn gly val asp leu arg asn ala ser his glu gln ala
        35                  40                  45
ala ile ala leu
    49
```

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 34:

thr ile thr ile gln lys gly pro gln gly leu gly phe asn ile
1 5 10 15
val gly gly
18

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

glu asp gly gln gly ile tyr val ser phe ile leu ala gly gly
1 5 10 15
pro ala asp leu gly ser glu leu lys arg gly asp gln ile leu
20 25 30
ser val asn asn val asn leu thr his ala thr his glu glu ala
35 40 45
ala gln ala leu
49

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

ser ile asn met glu ala val asn phe gly leu gly ile ser ile
1 5 10 15
val gly gln
18

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

ser asn arg gly gly asp gly gly ile tyr val gly ser ile met
1 5 10 15
lys gly gly ala ala val leu asp gly arg ile glu pro gly asp
20 25 30
met ile leu gln val asn asp val asn phe glu asn met thr asn
35 40 45
asp glu ala val arg val leu
50 52

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 74 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

```
glu val lys leu phe lys asn ser ser gly leu gly phe ser phe
 1               5                  10                  15
ser arg glu asp asn leu ile pro glu gln ile asn ala ser ile
            20                  25                  30
val arg val lys lys leu phe pro gly gln pro ala ala glu ser
            35                  40                  45
gly lys ile asp val gly asp val ile leu lys val asn gly ala
            50                  55                  60
ser leu lys gly leu ser gln gln glu ala ile ser ala leu
            65                  70                  74
```

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

```
val arg phe lys lys gly asp ser val gly leu arg leu ala
 1               5                  10                  14
```

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

```
gly gly asn asp
 1               4
```

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

```
val gly ile phe val ala gly ile gln glu gly thr ser ala glu
 1               5                  10                  15
gln glu gly leu leu gln glu gly asp gln ile leu lys val asn
            20                  25                  30
thr gln asp phe arg gly leu val arg glu asp ala val leu val
            35                  40                  45
leu val arg phe lys lys gly asp ser val gly leu arg leu ala
            50                  55                  60
```

WHAT IS CLAIMED IS:

1. A method for diagnosing muscular dystrophy in a mammal comprising determination of absence or decrease of neuronal-type nitric oxide synthase protein in a mammal skeletal muscle sample.

2. The method of Claim 1 comprising steps:
(a) obtaining the skeletal muscle biopsy sample; and
(b preparing the muscle sample cryosections; and
(c) detecting the presence or absence of nitric oxide synthase protein by immunofluorescence assay, by histochemical staining or biochemically.

3. The method of Claim 2 wherein the immunofluorescent assay comprises a detection with a primary antibody.

4. The method of Claim 2 wherein the detection of nitric oxide synthase is by histochemical staining for NADPH diaphorase.

5. The method of Claim 4 wherein the cryosections are incubated with NADPH and stained with nitroblue tetrazolium.

6. The method of Claim 5 wherein the presence of nitric oxide synthase is detected by a presence of blue color.

7. The method of Claim 1 wherein nitric oxide synthase is detected biochemically and the detection comprises analysis of the nitric oxide synthase protein by Western blotting.

8. A method for treatment of muscular dystrophy by restoration to a patient in need of such treatment of functional dystrophin or a functional fragment thereof able to bind neuronal nitric oxide to syntrophin in muscle sarcolemma.

9. The method of Claim 8 wherein neuronal nitric oxide is bound to syntrophin through its PDZ domain.

10. The method of Claim 9 wherein the method for treatment of muscular dystrophy involves a production of vectors encoding constructs or fragments thereof that assemble functional neuronal nitric oxide synthase/syntrophin/dystrophin complex.

11. A method for detection, prevention and treatment of neurodegenerative diseases by administering to a patient in need of such treatment an inhibitor of binding of neuronal nitric oxide synthase and a binding protein.

12. The method of Claim 11 wherein the binding protein is a protein identified as PSD-95 or PSD-93.

13. The method of Claim 12 wherein neuronal nitric oxide and the binding protein are bound through their respective PDZ domains.

14. An inhibitor of binding of neuronal nitric oxide synthase and a binding protein.

15. A diagnostic kit for detection of muscular dystrophy in a mammal muscle biopsy sample.

16. A binding protein identified as PSD-93 having sequences identified as SEQ ID NOs: 2-6.

17. A nucleic acid sequence identified as SEQ ID NO: 1 encoding a protein having sequences SEQ ID NOs: 2-6.

18. A binding assay to monitor interaction of N-methyl-D-aspartate receptors and neuronal nitric oxide synthase through binding interaction with binding proteins PDS-95 and PDS-93.

FIG. 2A

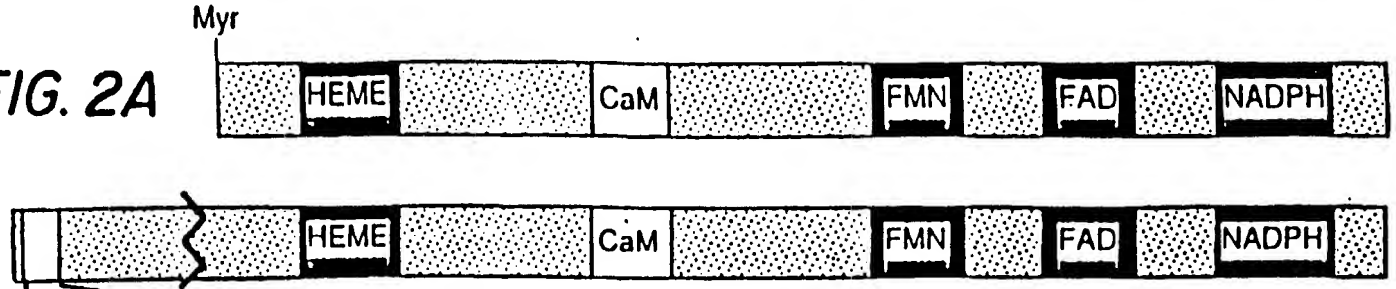


FIG. 2B

```
18    VRLFKRKVGGLGFLVK        ERVSKPPVIISDLIRGGAAEQSGLIQAGDIILAVNDRPLVDLSYDSALEVL   84   nNOS
69    VRIVKQEAGGLGISIKGG       RENHMPILISKIFRGLAAEQSRLLFVGDAILSVNGTDLRDATHDQAVQAL   136  T-SYN
15    VRVVKQEAGGLGISIKGG       RENRMPILISKIFPGLAADQSRALHLGDAILSVNGTDLRQATHDQAVQAL   82   SYN-I
313   RIVIHRGSTGLGFNIVGG        EDGEGIFISFILAGGPADLSGELHKGDQILSVNGVDLRNASHEQAAIAL   381  PSD-95
486   TITIQKGPQGLGFNIVGG  ....  EDGQGIYVSFILAGGPADLGSELKRGDQILSVNNVNLTHATHEEAAQAL   552  DLG
254   SINMEAVNFGLGISIVGQ  ..SNRGGDGGIYVGSIMKGGAAVLDGHIEPGDMILQVNDVNFENMTNDEAVRVL   323  DSH
1506  EVKLFKNSSGLGFSFSRED NLIPEQINASIVRVKKLFPGQPAAESGKIDVGDVILKVNGASLKGLSQQEAISAL  1579  PTP
98    VRFKKGDSVGLRLA  GGND        VGIFVAGIQEGTSAEQEGLLQEGDQILKVNTQDFRGLVRTEDAVLVL   151  ZO-1
                 **                          *  *        **  **  **          *  *
CON   VR  K    GLGFSI GG       RE    I IS I  GGAA OSG L  GD ILSVN  DL   T D A  VL
```

FIG. 2C

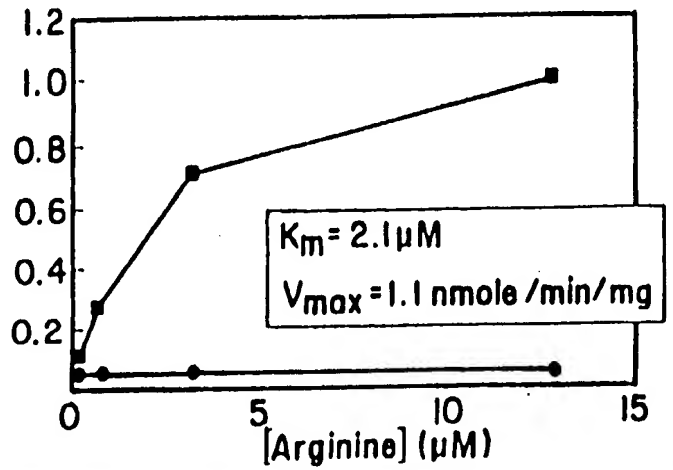


FIG. 2D

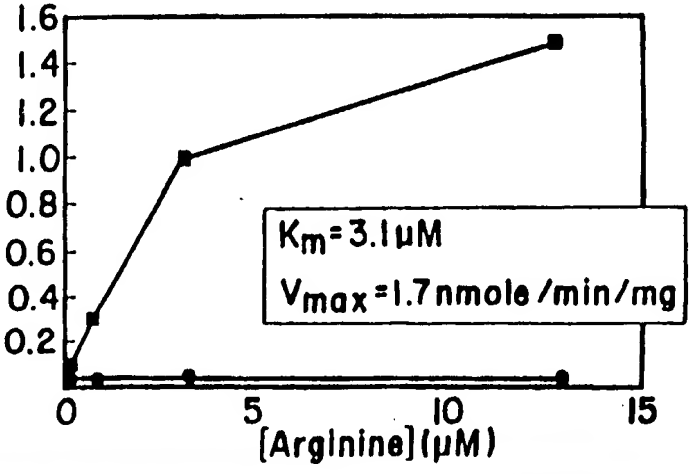


FIG. 4A

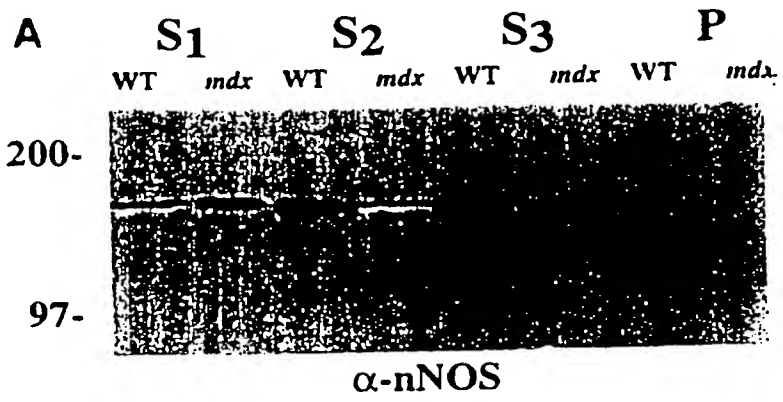


FIG. 4B

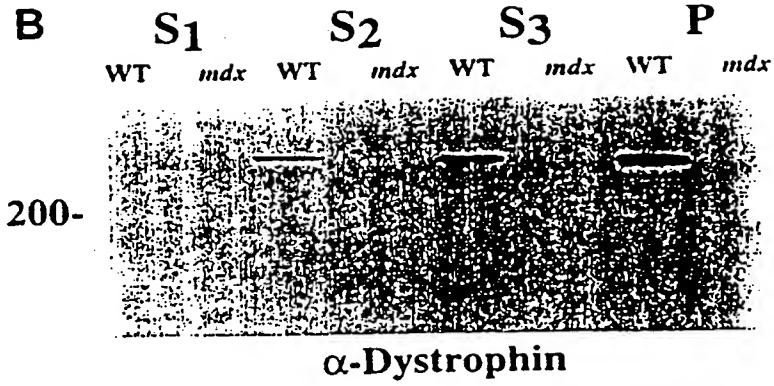


FIG. 6A

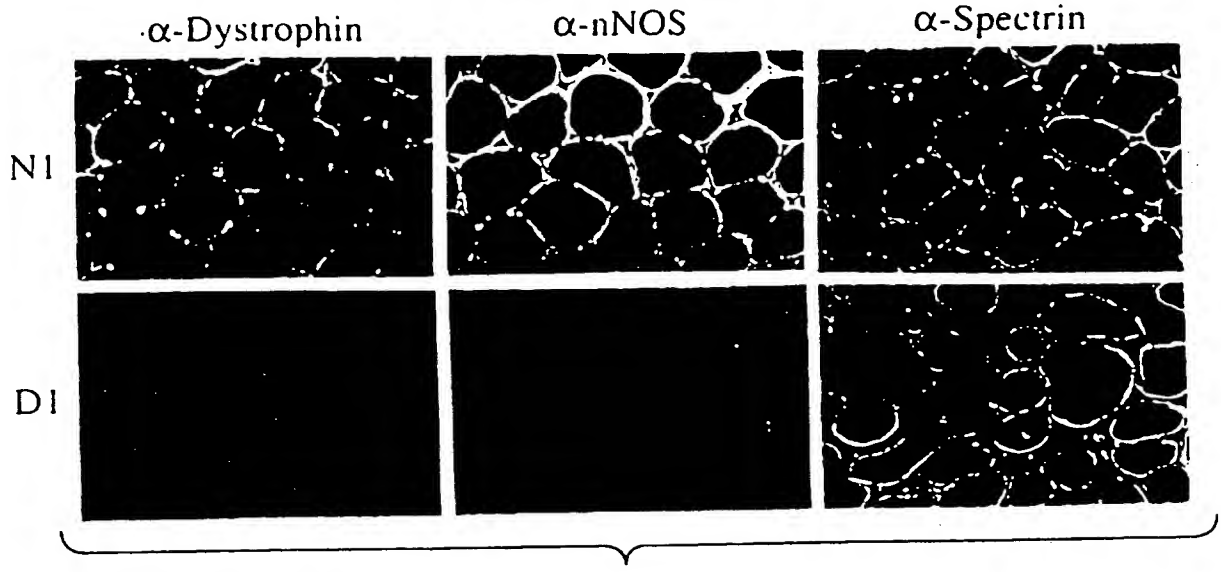


FIG. 6B

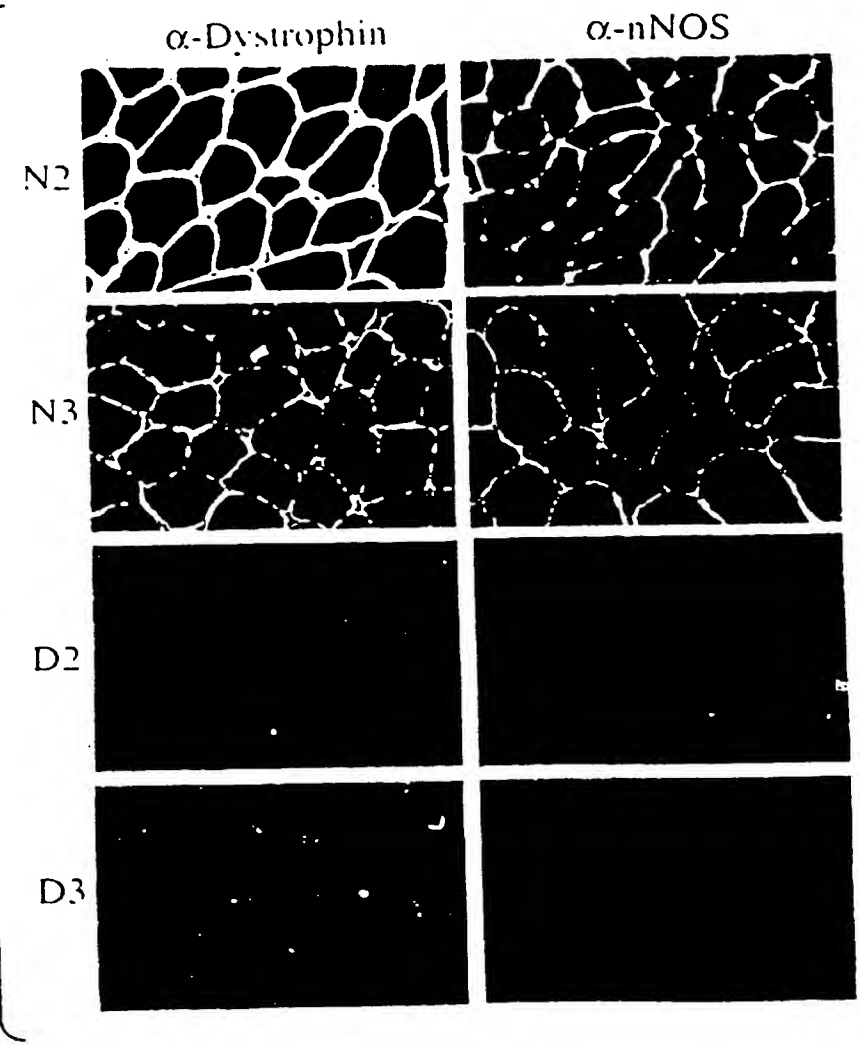


FIG. 7A

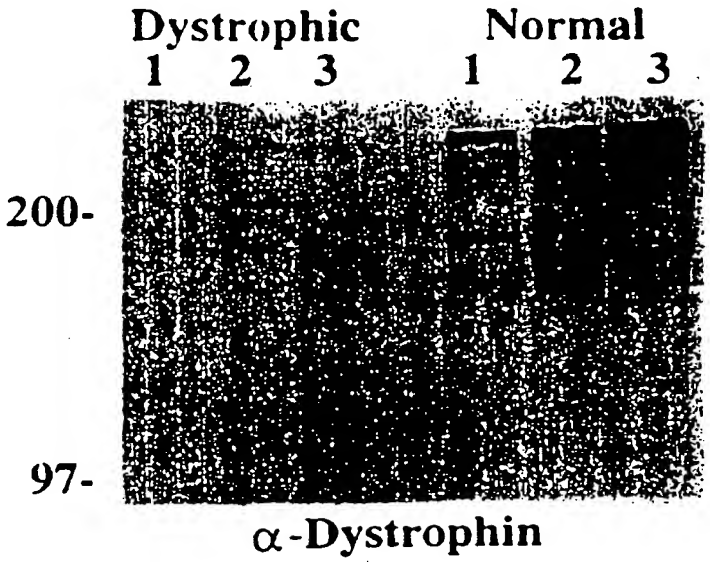


FIG. 7B

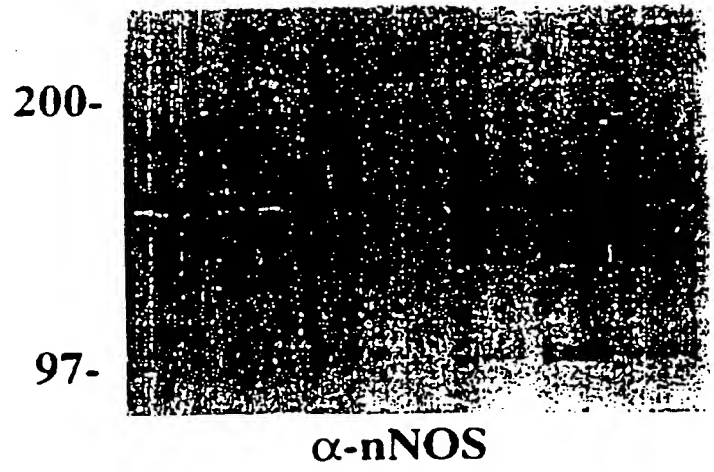


FIG. 7C

FIG. 8-2

FIG. 9-2

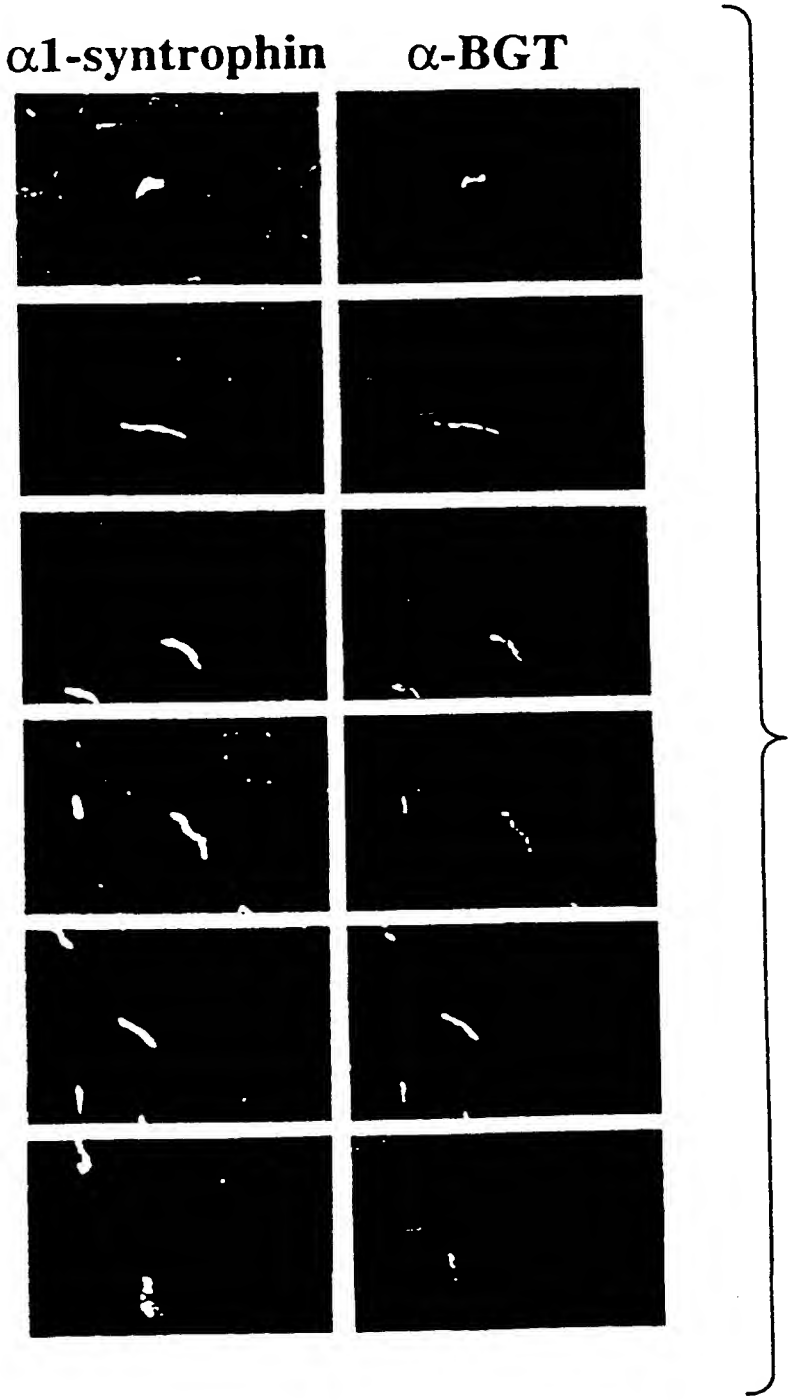


FIG. 11

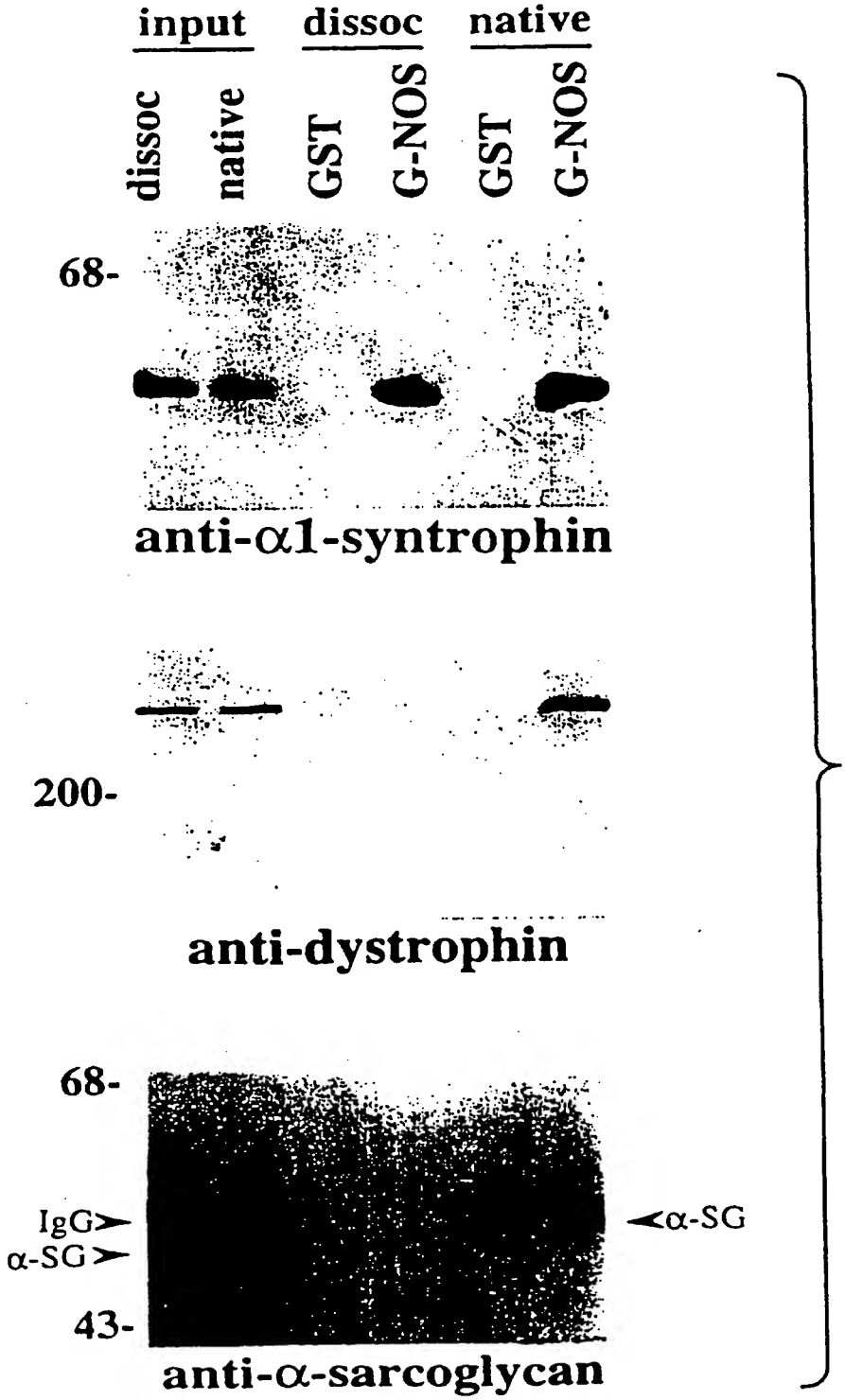


FIG. 12-2

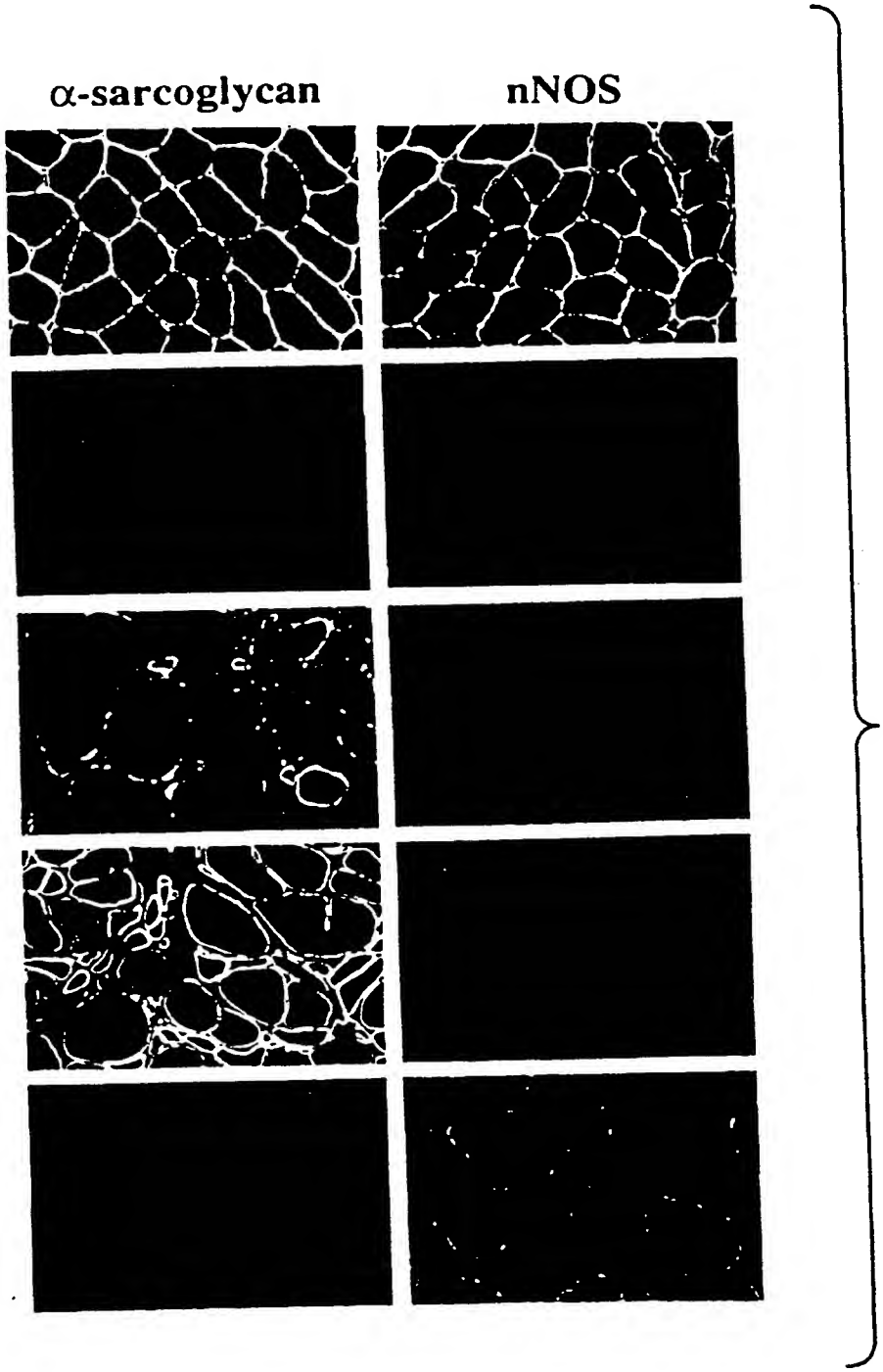


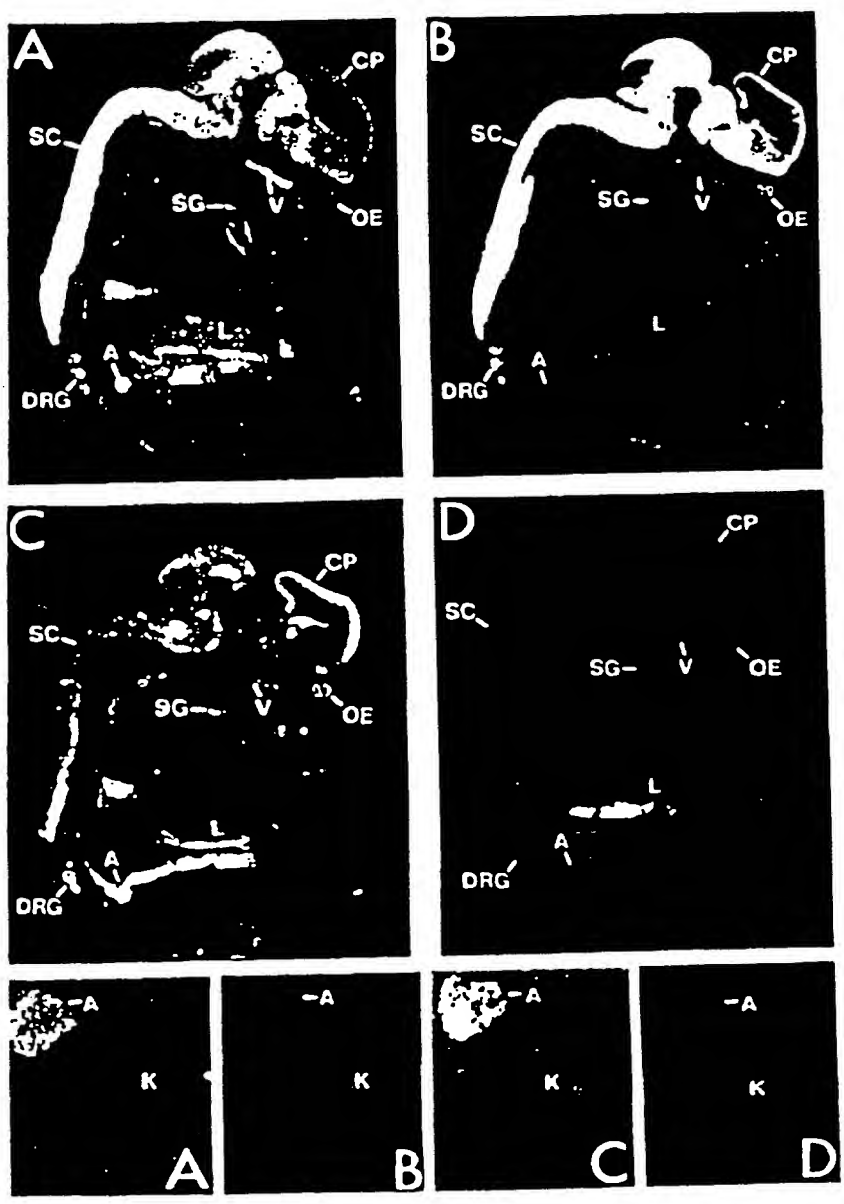
FIG. 14

```
                                                                                                                    96
PSD-93 MFFACYCALR TNVKKYRYQD EDGPH.DHSL PRLTHEVRGP ELVHVSEKNL SQIENVHGYV LQSHISPLKA SPAPIIVNTD TLDTIPY... VNGTEIEYEF
PSD-95 ..MDCLCIVT T..KKYRYQD EDTPPLEIISP AIIL..... ..........  .......... ......PNQA NSPPVIVNTD TLEAPGYELQ VNGTEGEMEY

                                                                                                                   196
PSD-93 EEITLERGNS GLGFSIAGGT DNPHIGDDPG IFITKIIPGG AAAEDGRLRV NDCILRVNEV DVSEVSHSKA VEALKEAGSI VRLYILRRRP ILETVVEIKL
PSD-95 EEITLERGNS GLGFSIAGGT DNPHIGDDPS IFITKIIPGG AAAQDGRLRV NDSILFVNEV DVREVTHSAA VEALKEAGSI VRLYVMRRKP PAEKVMEIKL

                                                                                                                   296
PSD-93 FKGPKGLGFS IAGGVGNQHI PGDNSIYVTK IMDGGAAQKD GRLQVGDRLL MVNNYSLEEV THEEAVAILK NTSDVVYLKV GKPTTIYMTD PYGPPDITHS
PSD-95 IKGPKGLGFS IAGGVGNQHI PGDNSIYVTK IIEGGAAHKD GRLQIGDKIL AVNSVGLEDV MHEDAVAALK NTYDVVYLKV AKPSNAYLSD SYAPPDITTS

                                                                                                                   396
PSD-93 YSPPMENHLL SGNNGTLEYK TSLPPISPGK YSPIPKHMLV EDDYTRPPEP VYSTVNKLCD KPASPRHYSP VECDKSFLLS TPYPHYHLGL LPDSDMTSHS
PSD-95 YSQHLDNEIS HSSYLGTDYP TAMTPTSPRR YSPVAKDLLG EEDIPR.... ..........  .......... .......... .......... ..........

                                                                                                                   495
PSD-93 QHSTATRQPS VTLQRAISLE GEPRKVVLHK GSTGLGFNIV GGEDGEGIFV SFILAGGPAD LSGELQRRKQ ILSVNGIHLP GDSHEQ.ALP LKGAGQTVTI
PSD-95 .......... .......... .EPRRIVIHR GSTGLGFNIV GGEDGEGIFI SFILAGGPAD LSGELRKGDQ ILSVNGVDLR NASHEQAAIA LKNAGQTVTI

                                                                                                                   595
PSD-93 IAQYQPEDYS RFEAKIHDLR EQMMNHSMSS GSGSLRTNQK RSLYVRAMFD YDKSKDSGLP SQGLSFKYGD ILNVINASDD EWWQARRVIQ DGDSEEMGVI
PSD-95 IAQYKPEEYS RFEAKIHDLR EQLMNSSLGS GTASLRSNPK RGFYIRALFD YDKTKDCGFL SQALSFRFGD VLHVIDAGDE EWWQARRVHS DSETDDIGFI

                                                                                                                   695
PSD-93 PSKRRVERKE RARLKTVKFN AKPGVIDSKR GQEDLILSYE PVTRQEINYT RPVIILGPMK DRINDDLISE FPDKFGSCVP HTTRPKRDYE VDGRDYHFVI
PSD-95 PSKRRVERRE WSRLKAKDWG SSSG....SQ GREDSVLSYE TVTQMEVHYA RPIIILGPTK DRANDDLLSE FPDKFGSCVP HTTRPKREYE IDGRDYHFVS

                                                                                                                   793
PSD-93 SREQMEKDIQ EHKFIEAGQY YDNLYGTSVQ SVRFVAERGK HCIMDVSGNA ISGYKLPSSI H..CYLIKPK SLEPLMEMNN GLMEEQAKKT YDRAIKLEQE
PSD-95 SREKMEKDIQ AHKFIEAGQY NSHLYGTSVQ SVREVAEQGK HCILDVSANA VRRLQ.AAHL HPIAIFIRPR SLENVLEINK RITEEQARKA FDRATKLEQE

                                                                                                                   844
PSD-93 FGEYFTAIVQ GDTLEDIYNQ CKLVIEEQSG PFIWIPSKEK L
PSD-95 FTECFSAIVE GDSFEEIYHK VKRVIEDLSG PYIWVPARER L
```

SEQ ID NO. 11

SEQ ID NO. 12

FIG. 15C



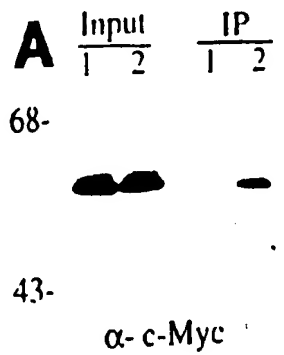


FIG. 17A

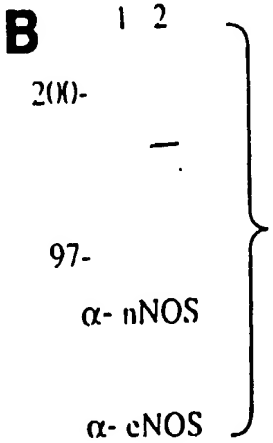


FIG. 17B

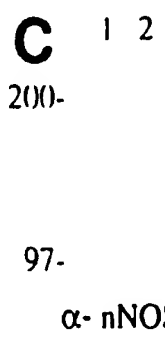


FIG. 17C

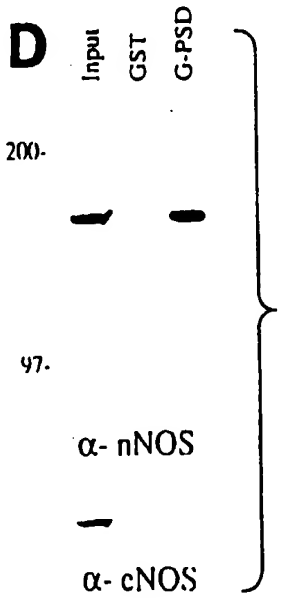


FIG. 17D

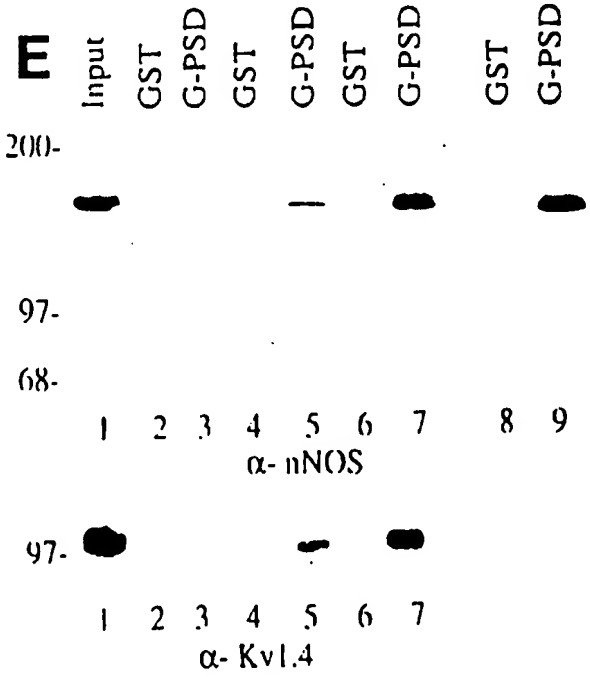


FIG. 17E

FIG. 19A

FIG. 19B

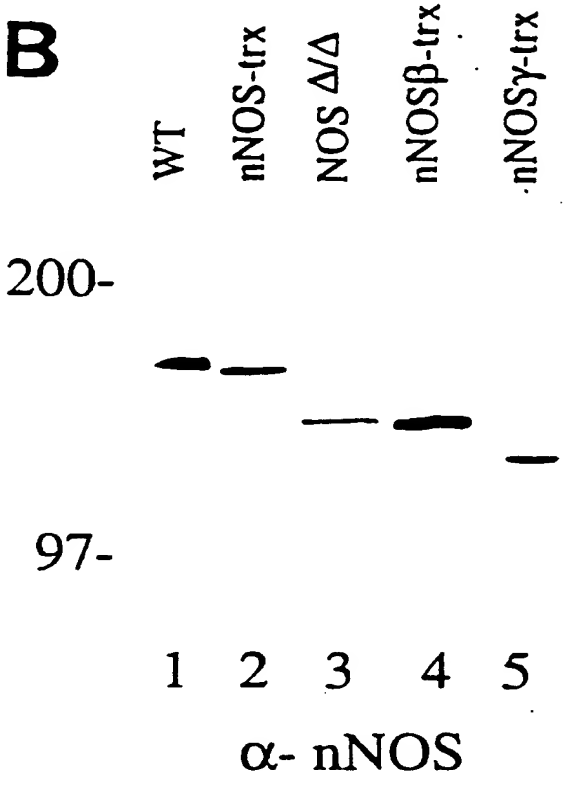


FIG. 19C

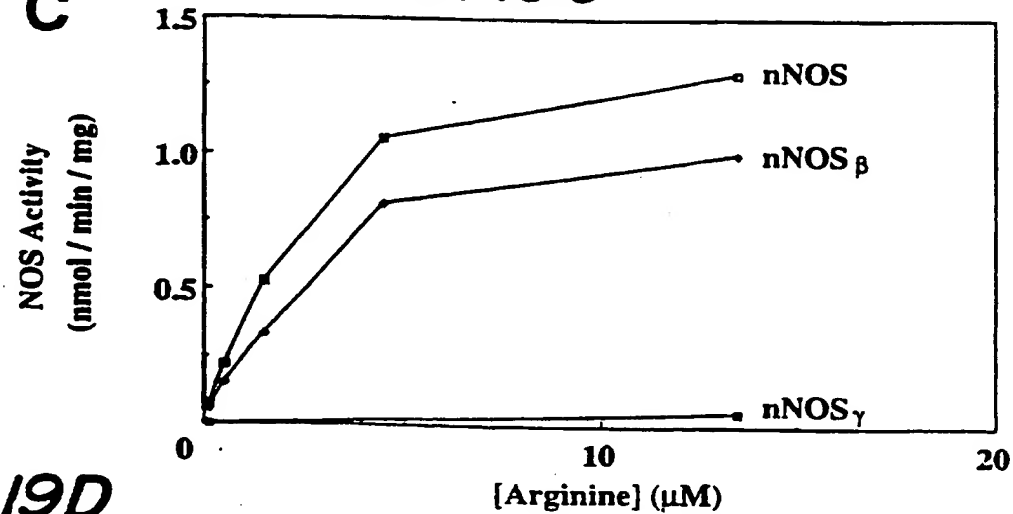


FIG. 19D

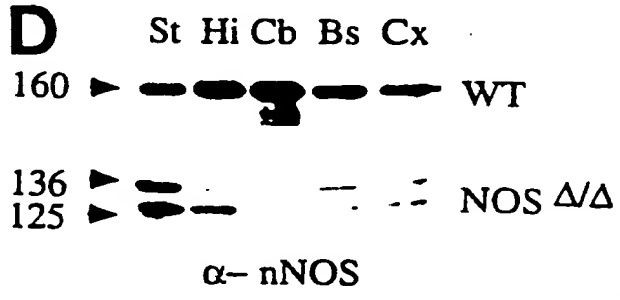


FIG. 19E

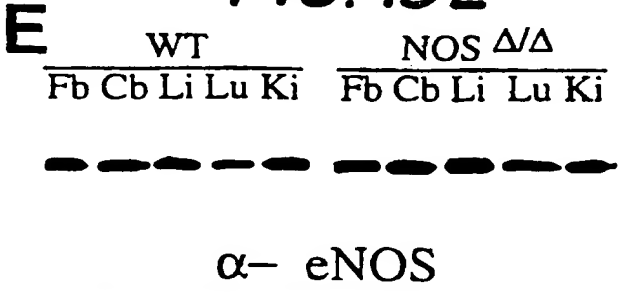


FIG. 21A

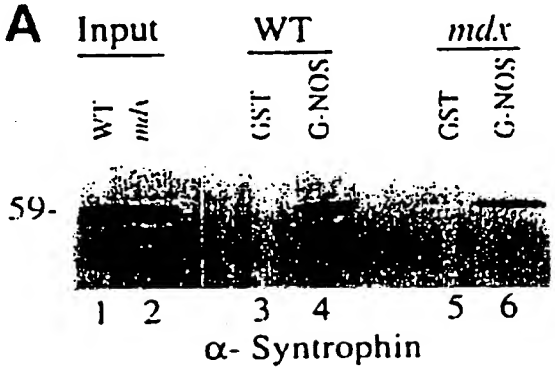


FIG. 21B

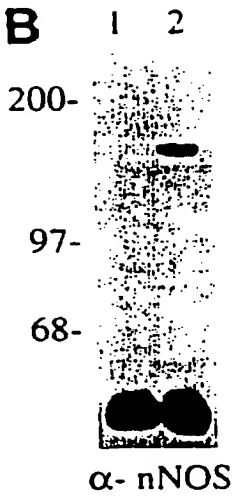


FIG. 21C

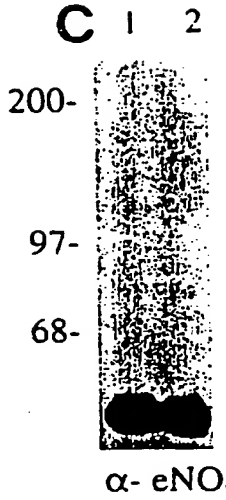
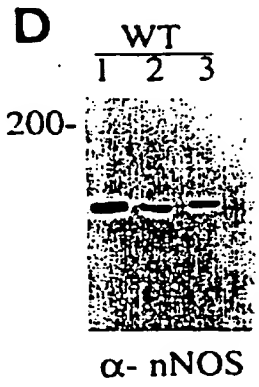


FIG. 21D



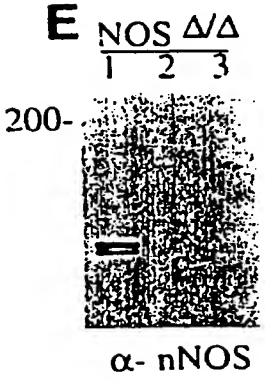


FIG. 21E

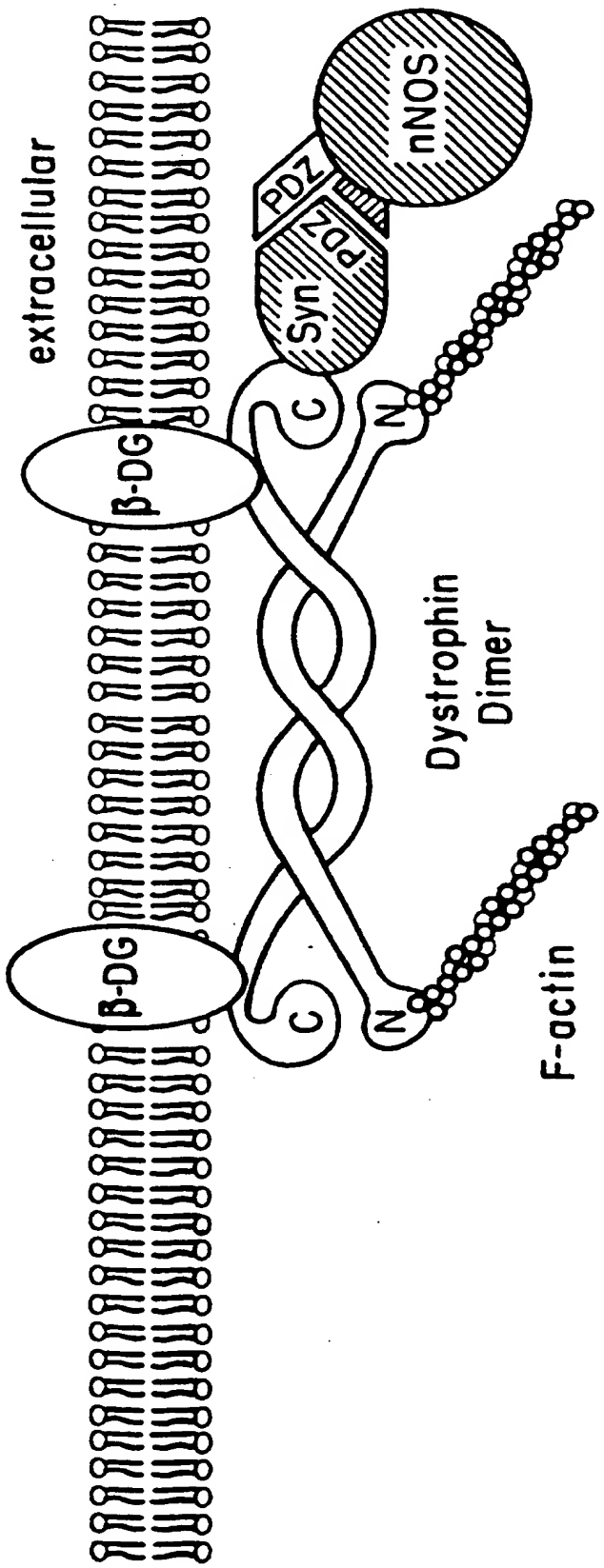


FIG. 22D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03897

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CAMPBELL. Three Muscular Dystrophies: Loss of Cytoskeleton-Extracellular Matrix Linkage. Cell. 10 March 1995, Vol. 80, pages 675-679, see entire document.	8-14, 16-18
Y	PETERS et al. β2-Syntrophin: localization at the neuromuscular junction in skeletal muscle. NeuroReport 15 August 1994, Vol. 5, No. 13, pages 1577-1580, see entire document.	8-14, 16-18
Y	ADAMS et al. Mouse α1- and β2-Syntrophin Gene Structure, Chromosome Localization, and Homology with a Discs Large Domain. Journal of Biological Chemistry. 27 October 1995, Vol. 270, No. 43, pages 25859-25865, see entire document.	9, 10
Y	CHO et al. The Rat Brain Postsynaptic Density Fraction Contains a Homolog of the Drosophila Discs-Large Tumor Suppressor Protein. Neuron, November 1992, Vol. 9, pages 929-942, see entire document.	12, 13, 16
Y	US 5,266,594 A (DAWSON et al.) 30 November 1993, see entire document.	18
Y	US 5,466,676 A (BOOTH et al.) 14 November 1995, see entire document.	8-14, 18
Y	US 5,260,209 A (CAMPBELL et al.) 09 November 1993, see entire document.	8-14, 18
Y	DAWSON et al. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc. Natl. Acad. Sci. USA. July 1991, Vol. 88, pages 6368-6371, see entire document.	11, 12, 16, 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03897

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Automated Patent System - files USPAT, JPOABS, and EPOABS
DIALOG files - MEDLINE, BIOSIS, EMBASE
Search terms: neuron(s, al), nitric, oxide, synthase(s), PSD-95, PSD-93, synthrophin, dystrophin, biopsy, cryostat, cryosection(s), muscular dystrophy, receptor(s), NMDA, aspartate, N-methyl-D-aspartate, Duchenne, post, synaptic, density, pdz, glutamate

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I. Claim(s) 1-7, 15, drawn to a method of diagnosing muscular dystrophy (MD) by detecting neural nitric oxide synthase (the first claimed method of use), hereinafter nNOS; and a kit for detecting MD

Group II. Claim(s) 8-9, drawn to a method of treating MD that binds nNOS to syntrophin comprising, administration of functional dystrophin or a fragment of dystrophin; i.e., a second process that uses a different protein as that active agent than in Group I which uses nNOS.

Group III. Claim(s) 10, drawn to a method of treating MD by administration of vectors encoding constructs that effect assemly of a functional multiprotein nNOS/syntrophin/dystrophin complex.

Group IV. Claim(s) 11-14, drawn to a method of detecting, preventing, and treating a neurodegenerative disease by inhibiting binding of nNOS to a binding protein by administering an inhibitor of binding and the inhibitor.

Group V. Claim(s) 16, drawn to a binding protein. The binding protein is not nNOS.

Group VI. Claim(s) 17, drawn to a nucleic acid polymer encoding the a binding protein. The binding protein is not nNOS.

Group VII. Claim(s) 18, drawn to a binding assay that monitors interaction of nNOS and aspartate receptors via interaction with binding proteins.

The inventions listed as Groups I through VII do not relate to a single inventive concept under PCT Rule 13.1 because,under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The Bredt et al. (1991) Nature 351: 714-714 reference discloses that nNOS was known and that its gene had been cloned. Thus, the special technical feature of Group I, nNOS, does not define a contribution over the prior art. Moreover, Group II administers a dystrophin or a fragment thereof. Dystrophin is not the protein nNOS used in Group I.